Gene expression studies in Atlantic salmon (*Salmo salar* L.)

Effects of peroxisome proliferator-activated receptor agonists

by

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Thesis for the degree *candidata pharmaciae*

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FOREWORD

The work on this master thesis was carried out at the School of Pharmacy, Department of Pharmaceutical Biosciences at the University of Oslo, in the period of December 2006 to November 2007.

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This thesis marks the end of my education at the University of Oslo, a journey that have taught me a lot along the way.

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ABSTRACT

Since their discovery in the early 1990s, peroxisome proliferator-activated receptors (PPARs) have become an extremely important set of targets for drug discovery. The ligand-induced transcription factors that regulate the transcription of target genes in response to specific ligands, keeps on broadening its repertoire as new knowledge are uncovered. PPARs are nuclear lipid-activated receptors that control a vast variety of genes in several pathways of lipid metabolism. This includes fatty acid transport, uptake by the cells, intracellular binding and activation, as well as catabolism (β-oxidation and ω-oxidation) or storage. They are important pharmacological targets of treatment of obesity, diabetes and lipid disorders.

Although, PPARs are among the most studied nuclear receptors, there is little knowledge of their activity and functions in fish. Atlantic salmon- *Salmo salar* L. belongs to the family Salmonidae (Salmonids) and the order Salmoniformes. Norway have traditionally been farming Atlantic salmon since the early 1970s, and is today one of the major producers of farmed salmon for human consumption. The quality of fish depends much on the mechanisms that keep the fish healthy. The adipose regulations in fish are still unknown, and it is of great interest that they are investigated.

One of the goals of this thesis was to study PPAR gene expression in Atlantic salmon. We compared PPAR tissue distribution in various fish tissue and cell lines. The tissue distribution of PPARs in salmon was comparable to what has been described for mammals, a higher concentration in tissues where adipose metabolism is more relevant. We also exposed SHK-1 and ASK cells (Atlantic salmon head kidney cells) to PPAR agonist treatment and found that when activating PPARγ, an up-regulation of target genes like SR-BI and CD 36 where seen. These target genes play a key role in regulation of cholesterol homeostasis and have previously been shown to be up-regulated by PPARγ in mammals. To further investigate PPARγ, we performed transfection studies. Although, we obtained low transfection efficiency, the findings showed same trend in PPAR transcription activity regulation.

Highly specific antibodies against fish antigens are rare. We therefore performed assays to test a novel anti salmon-PPARγ antibody for specificity, applying methods such as immunostaining and western blotting. If the PPARγ antibody proved specific, it would

provide us with an important tool in PPAR studies. Unfortunately this was not the case in our study.

Our results of PPAR activity in Atlantic salmon head kidney cells agreed in many aspects with previous findings in mammalian cells. However, there were low effects of ligand treatment, and it came apparent during this work that PPAR agonists could have toxic effects on SHK-1 and ASK cells in the concentrations employed here.

TABLE OF CONTENTS

ABBREVIATIONS

ACO	Acyl-Coenzym A oxidase
AF-1	Activation function-1
AF-2	Activation function-2
APS	Ammonium persulphate
ASK	Atlantic salmon head kidney cell
BSA	Bovine serum albumine
CD 36	Scavenger receptor subclass B member
CE	Cholesteryl esters
cDNA	complementary DNA
DBD	DNA-binding domain
DMSO	Dimethyl sulfoxide
DR	Direct repeats
EF1-α	Elongation factor 1-α
ER	Everted repeats
FA	Fatty acid
FBS	Foetal Bovine Serum
FCS	Foetal Calf serum
GFP	Green fluorescence protein
HDL	High density lipoprotein
HRP	Horse reddish protein
IR	Inverted repeats
LBD	Ligand-binding domain
LDL	Low density lipoprotein
LPL	Lipoprotein lipase
LXR	Liver X receptor
MeOH	Methanol
mRNA	Messenger RNA
(n)	nucleotid
NR	Nuclear receptor
PBS	Phosphate Buffered Saline
PBS-T	PBS+ Tween 20
PFA	Paraformaldehyde
PPAR	Peroxisome proliferator-activated receptor

PPAR-α	Peroxisome proliferator-activated receptor-alpha
PPAR-β	Peroxisome proliferator-activated receptor-beta
PPAR-γBoth	Peroxisome proliferator-activated receptor-gamma Both
PPAR-γLong	Peroxisome proliferator-activated receptor-gamma Long
PPAR-γShort	Peroxisome proliferator-activated receptor-gamma Short
PPRE	PPAR response element
PUFAs	Polyunsaturated fatty acids
RE	Response element
RT-PCR	Real-Time Polymerase chain reaction
RXR	Retinoid X receptor
SHK-1	Atlantic salmon head kidney-1 cell
SR-BI	Scavenger receptor class B type I
TEMED	(N,N,N,N,-tetramethyl-Ethylendiamin)
TF	Transcription factor
TNF-α	Tumor necrosis factor-α
TTA	Tetradecylthioacetic acid
TZD	Thiazolidinediones
UV	Ultra violet
2-ME	β-mercaptoethanol
Δ-5	Delta five fatty acid desaturase
Δ-6	Delta six fatty acid desaturase
18S	18S ribosomal RNA

1 GENERAL INTRODUCTION

1.1 Transcriptional regulation

There are numerous factors involved in the transcription of genes that control the metabolism in any organism. In the human genome, these transcription factors (TFs) make up for approximately 10 percent of all known protein coding genes. This relatively large number of TFs indicates a complex and tight regulation of metabolic control (Lander, Linton et al. 2001). The expression of genes is dependent on the TFs binding to specific DNA sites called response elements (RE), in the promoter (regulatory) area of the target genes (Lee and Young 2000). The promoter is localized in front of the coding sequence of a gene which consists of a transcription initiation site, often a TATA box and / or an initiator element that facilitate the assembly of the general transcription factors that are required for specific promoter binding by RNA polymerase II (Lee and Young 2000; Levine and Tjian 2003; Smale and Kadonaga 2003).

1.2 Nuclear receptors

Nuclear receptors are a superfamily of DNA-binding transcription factors that are similar in structure, and believed to have evolved from a common ancestor. The nuclear receptors regulate the expression of target genes that have effect on processes like reproduction, development and general metabolism. The nuclear receptor superfamily includes the classic endocrine receptors that mediate the actions of steroid hormones, thyroid hormones, and the fat-soluble vitamins A and D, as well as a large number of so-called orphan nuclear receptors. The latter group is divided in two subgroups; the adopted orphan receptors, which have known ligands, and the orphan receptors where the ligands are still unknown. Members of the adopted orphan receptor group are also referred to as “sensor” receptors (Desvergne, Michalik et al. 2006), which include for example, receptors for fatty acids, liver X receptor (LXR) and retinoid X receptor (RXR) (figure 1.1) (Evans 1988).

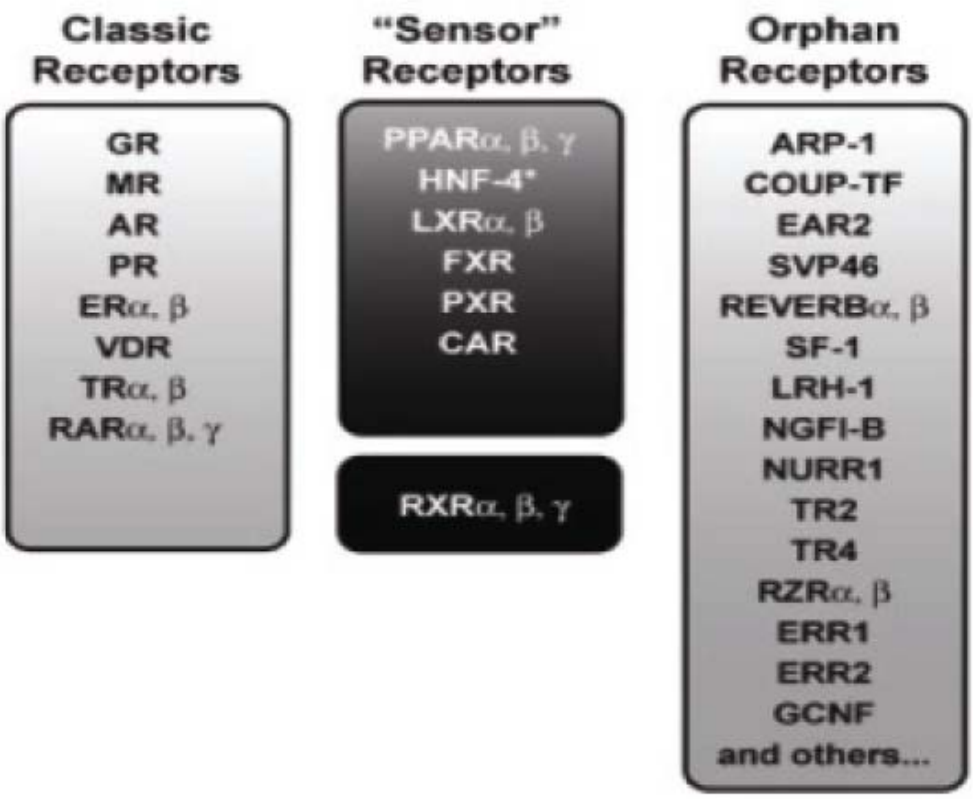

Figure 1.1 The nuclear receptor superfamily. The classic hormone receptors bind with high affinity to molecules. Their activation is associated with many metabolic adjustments; they are key factors of endocrine homeostasis. The "sensor" receptors are sensors of the metabolic status, they respond both to exogenous signals as well as metabolites generated in the organism, and are responsible for the metabolic adaptation at on cellular, organ, and whole organism level. The orphan receptors display the structural characteristics of nuclear receptors including a ligand binding domain. Thus, no ligand has so far been identified for these receptors (Desvergne, Michalik et al. 2006).

1.2.1 Nuclear receptor structure

Nuclear receptors have a ligand-independent transcription activation domain at the NH_2 – terminal, that is called A/B domain. Since this region is important for transcription activation, it is often referred to as the activation function-1 (AF-1) (Lees, Fawell et al. 1989; Tora, White et al. 1989). A DNA-binding domain (DBD or C domain) is found in the core of nuclear receptor (NR) containing two highly conserved 'zinc-finger' domains that target the receptor to specific DNA response element (RE). The D domain is a variable hinge region that gives the receptor flexibility and allows simultaneous receptor dimerization and DNA binding. Adjacent to the hinge region is the ligand-binding domain (LBD or E/F domain) that mediates ligand binding, dimerization, interaction with heat shock proteins, nuclear

localization and transactivation. Located at the COOH-terminal is the activation function-2 (AF-2) domain that binds co-regulators (Chawla, Repa et al. 2001). The nuclear receptors bind to REs at their respective target genes that consist of one or two consensus core half site sequences in the promoter region. Binding occurs either as a monomer, homodimer or a heterodimeric complex with another NR; often with RXR (receptor for 9-*cis*-retinoic acid) to the consensus sequence AGGTCA. The consensus sequences are arranged as direct-(DR), inverted- (IR) or everted (ER) repeats separated by 1-6 nucleotides (n). Ligand-binding induces conformational changes in the nuclear receptor molecule. This cause translocation to the nucleus and changes in transcriptional activity of target genes by increasing or decreasing receptor activity (figure 1.2) (Smirnov 2002).

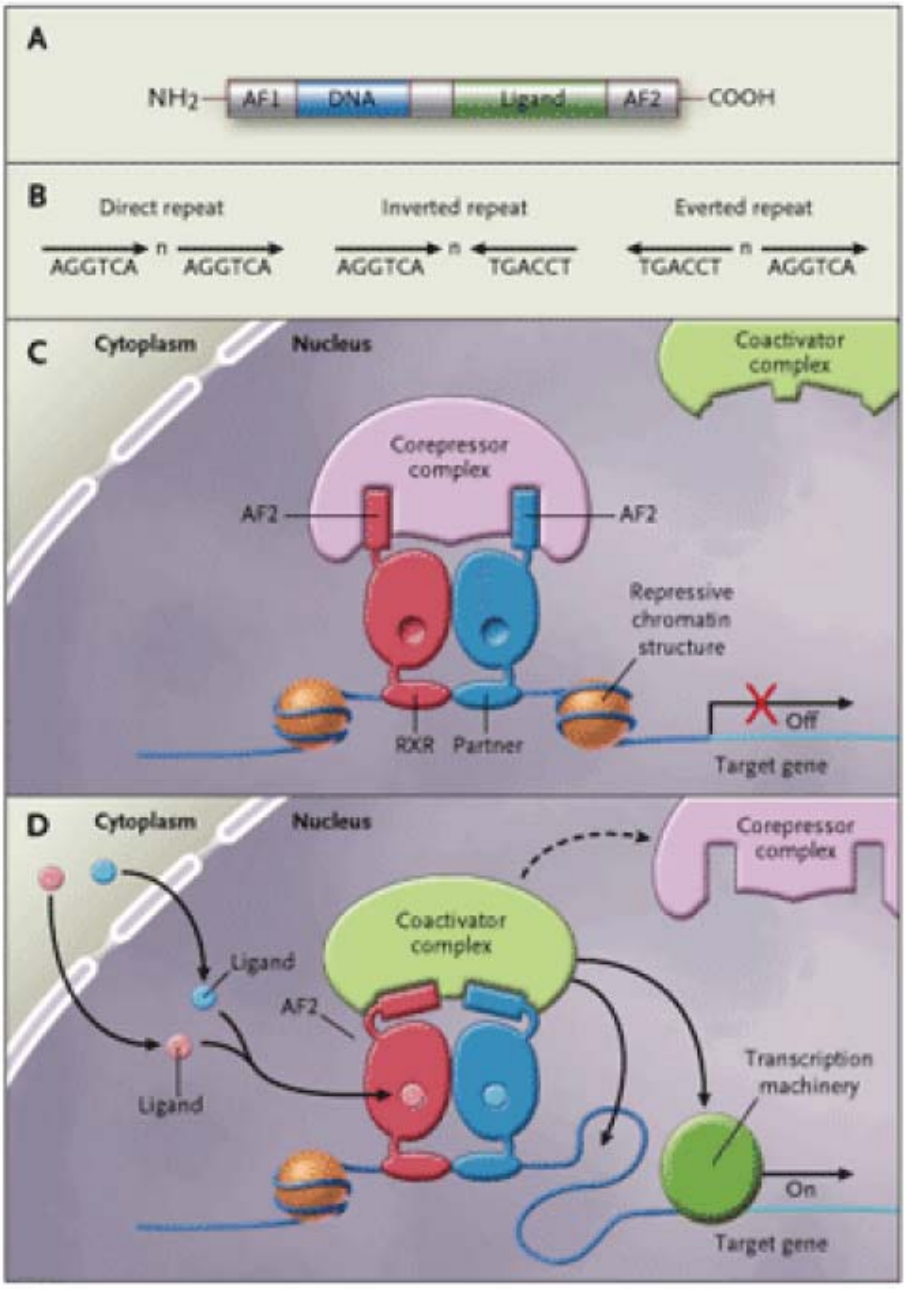

Figure 1.2 Nuclear Receptors as Ligand-Dependent Transcription Factors. (A) Shows the structure of the nuclear receptor, which includes N-terminal activation function 1 (AF-1), DNA binding, ligand binding, and C-terminal AF-2 domains. Responsive elements in the nuclear receptor can be configured as either direct, inverted, or everted repeats of the hexad core sequence AGGTCA (B). The number of nucleotides between the two core elements (n) confers additional specificity. (C and D) RXR heterodimers constitutively bind to response elements in the promoter regions of target genes. In Panel C, in the absence of agonist ligand, the AF-2 domain conformation promotes receptor interaction with co-repressors. The multiple-subunit co-repressor complex stabilizes repressive local chromatin structure and blocks access of the transcription machinery (red X) to the promoter. In Panel D, ligands diffuse across the nuclear membrane and bind to receptor ligand-binding domains. Ligand binding triggers a conformational change in the AF-2 domain, which destabilizes co-repressor interaction and promotes co-activator binding. Multiple-subunit co-activator complexes activate local chromatin structure and recruit the transcription machinery to the promoter, where target-gene transcription commences (adapted from (Shulman and Mangelsdorf 2005).

1.3 Peroxisome proliferator-activated receptors (PPARs)

Peroxisome proliferator-activated receptors are ligand-regulated transcription factors and belong to the nuclear receptor superfamily. PPARs are some of the most examined nuclear receptor because of their involvement in regulation of lipid homeostasis (Desvergne and Wahli 1999).

1.3.1 Structure and activity of PPARs

Like the other members of the nuclear receptor superfamily, PPAR proteins are also composed of several different domains (figure 1.3).

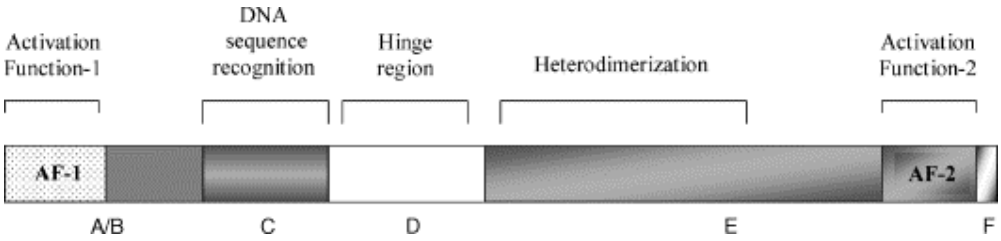

Figure 1.3. Structure of a PPAR molecule. (Blanquart, Barbier et al. 2003)

1.3.2 PPAR binding

The transcription of PPAR is regulated by their specific target genes binding to a PPAR response element (PPRE) located in the regulatory area of the gene (figure 1.4). This DNA element is a DR1 which means that the RE consists of a direct repeat of the consensus sequence, AGGTCA half site spaced by one (or two) nucleotides (Marx, Duez et al. 2004).

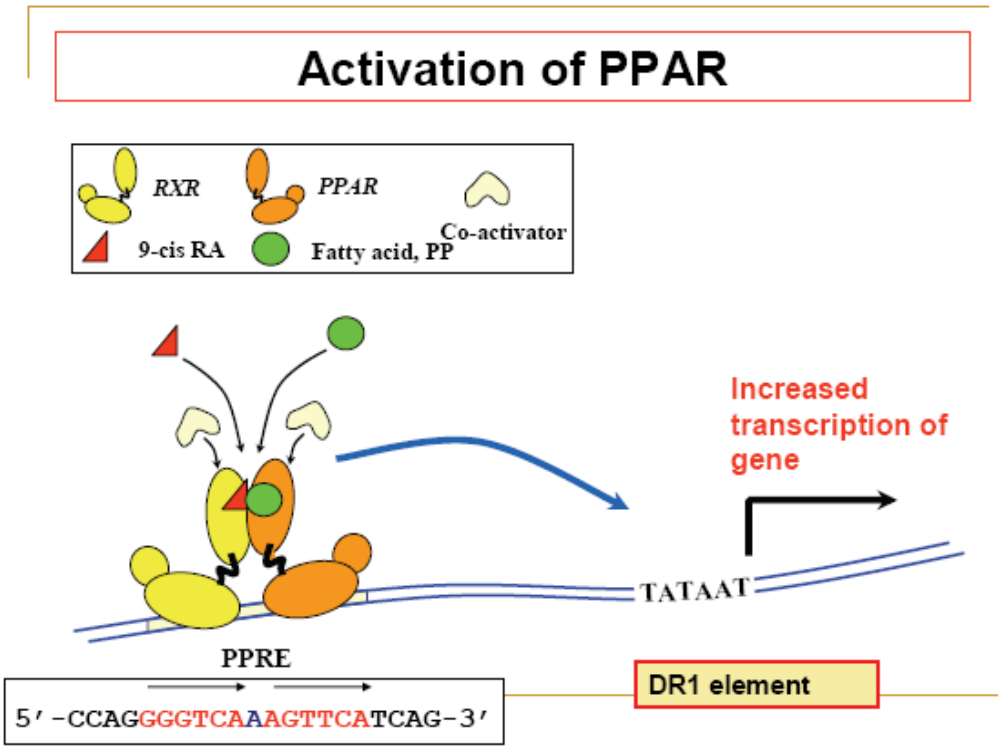

Figure 1.4 When PPAR is activated by ligands, PPAR heterodimerizes with RXR and regulate transcription by binding to a specific PPAR response element (PPRE) in the promoter in the target gene.

A common feature among some of the NRs is that they transcriptionally regulate their target genes as heterodimers with retinoid X receptors (RXRs). The PPARs are activated by specific ligands which bind to their LBD. PPARs are activated by a variety of endogenous compounds such as fatty acids and eicosanoids, but more potent are the synthetic PPAR ligands; fibrates and thiazolidinediones (Berger and Moller 2002). The activation of PPARs depends greatly on their particular ligand binding properties, which have important consequences for the PPAR biology. For instance, PPARs accommodates several

types of ligand, the molecules that specifically bind to PPAR do so with various affinities and there is some overlap in ligand recognition by the different PPAR isotypes (some ligand binds to more than one isotype)(Desvergne and Wahli 1999). The PPARs are important pharmacological targets for treatment of obesity, diabetes and lipid disorders.

1.4 PPAR isotype

There are three isotypes of peroxisome proliferator-activated receptors, designated PPARα (NR1C1), PPARβ/δ (NR1C2) and PPARγ (NR1C3)(Issemann and Green 1990). Although all three isoforms share similar protein sequence and structure, they differ in tissue distribution, ligand selectivity and biological actions (figure 1.5) (Desvergne and Wahli 1999; Escher, Braissant et al. 2001).

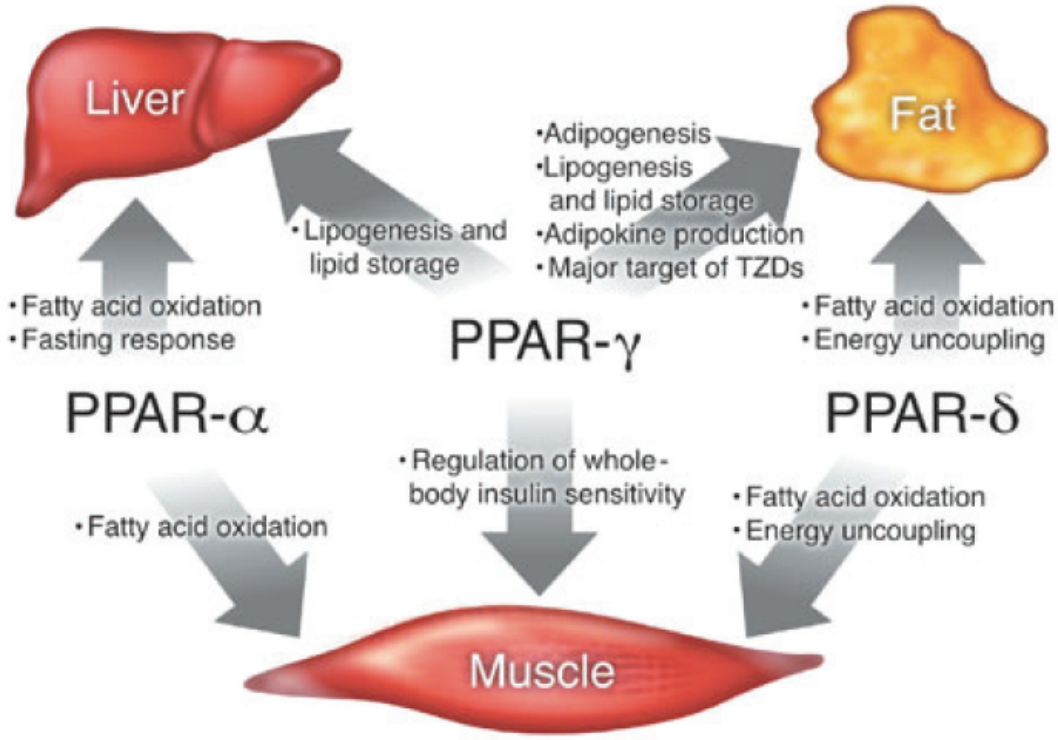

Figure 1.5 Metabolic integration by PPARs. The three PPAR isoforms regulate lipid and glucose homeostasis through coordinated activities in liver, muscle and adipose tissue. Adopted from (Evans, Barish et al. 2004)

1.4.1 PPARα

PPARα is expressed in tissues with high β-oxidation activity corresponding to its role in regulating the oxidation of fatty acids, liver tissue, brown adipose tissue, muscle, heart and kidney tissues (Ristow, Muller-Wieland et al. 1998). During a prolonged fast (overnight or starvation) fatty acids are released from the adipose tissue and transported into the liver where PPARα is heavily induced. PPARα is irreplaceable for a proper response to fasting in liver. (Kersten, Seydoux et al. 1999). Activation of PPARα by fatty acids promotes hepatic fatty acids oxidation to generate ketone bodies and by this provide an energy source for peripheral tissues. The importance of this is seen in PPARα- null mice, as they are unable to meet the energy demands during fasting and consequently suffers from hypoglycaemia, hyperlipidemia, hypoketonemia and fatty liver (Kersten, Seydoux et al. 1999). The induction of fatty acids oxidation by PPARα activation improves plasma lipid profiles. PPARα selective agonists like fibrates are often used to treat hypertriglyceridemia, as it lower plasma triglycerides, reduce adiposity and improve hepatic and muscle steatosis, which consequently improve insulin sensitivity (Guerre-Millo, Gervois et al. 2000; Chou, Haluzik et al. 2002).Other ligands are polyunsaturated fatty acids, including DHA and EPA, oxidised phospholipids and lipoprotein lipolytic products (Marx, Duez et al. 2004)

1.4.2 PPARγ

PPARγ is found in high concentrations in white adipose tissue and macrophages (Fajas, Auboeuf et al. 1997). The high expression of PPARγ in white adipose tissue is linked to its role as a crucial regulator of the adiopogenesis, the process of preadipocytes maturing into adipocytes(Tontonoz, Hu et al. 1994; Rosen, Walkey et al. 2000). This is supported by the findings that PPARγ knockout mice fail to develop adipose tissue (Barak, Nelson et al. 1999). PPARγ ligands are naturally occurring fatty acids derivatives, prostaglandin derivatives and synthetic compounds such as the antidiabetic thiazolidinediones (TZDs). When acting as direct agonists for PPARγ, these drugs reduce peripheral insulin resistance and thus lower blood glucose levels in patients with type 2 diabetes. The activation of PPARγ results in a reduced release of FAs and insulin resistance-mediating adiopocytokines, such as tumor necrosis factor- α (TNF-α), leptin or resistin, and an increased production of the antidiabetic adiponectin, which promotes fatty acid oxidation and insulin sensitivity in liver and muscle tissue (Berg, Combs et al. 2001; Yamauchi, Kamon et al. 2001; Marx, Duez et al. 2004).

1.4.3 PPARβ/δ

PPARβ/δ has earlier received less attention than the other PPARs, mainly because of its ubiquitous expression and until recently unknown functions. It is activated by polyunsaturated FAs (PUFAs), prostaglandins and synthetic compounds. Similar to PPARα, PPAR β/δ it plays an important role in the regulation of glucose and lipid levels in plasma, by stimulating FA oxidation in heart and skeletal muscle. The synthetic PPARβ/δ agonist GW501516 has shown its ability to lower plasma triglyceride level in obese monkeys, while raising high-density lipoprotein levels (Oliver, Shenk et al. 2001). Studies conducted with GW501516, reveal PPARβ/δ as a powerful regulator of fatty acid catabolism and energi homeostasis (Peters, Lee et al. 2000; Barak, Liao et al. 2002). Activated receptors induces genes that are required for fatty acids catabolism and adaptive thermogenesis (Wang, Lee et al. 2003). Treatment with PPARβ/δ agonist significant retards weight gain in animals and it also improves insulin resistance induced by high-fat diet, probably as a consequence of increased fat burning by muscle and the overall improvement in systemic lipid metabolism (Luquet, Lopez-Soriano et al. 2003).

1.5 PPAR ligands

1.5.1 Fenofibrates

Fenofibrates belongs to a class of hypolipidemic drugs, which are used in treatment of dyslipidemic patients. The fenofibrates act as peroxisome proliferator-activated receptor α (PPAR α) ligands, which are involved in the regulation of the expression of a number of genes which are critical for lipid and lipoprotein metabolism (figure 1.6) (Staels, Dallongeville et al. 1998). The known target genes of PPAR α include acyl-coenzym A oxidase (ACO), enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase, thiolase used for peroxisomal fatty acid β-oxidation, carnitine palmitoyltransferase I used for mitochondrial fatty acid β-oxidation, apolipoprotein C-III and lipoprotein lipase (LPL) used for the hydrolysis of plasma triglycerides (Auwerx, Schoonjans et al. 1996).

To some extent, fenofibrates seem to regulate energy homeostasis. When there is an excessive energy intake, an increase in the concentrations of plasma triglycerides, cholesterol and lipids accumulation in adipose tissue is observed (Chaput, Saladin et al. 2000). Fenofibrates increase hepatic fatty acid oxidation and decrease hepatic triglycerides; this may inhibit an increase in

body weight, which suggests that PPARα may have a role in the regulation of obesity. Studies have shown that PPARα-deficient mice have abnormal triglyceride and cholesterol metabolism. These mice eventually become obese with increasing age (Costet, Legendre et al. 1998)

1.5.2 Rosiglitazone

Rosiglitazone is a thiazolidinedione (TZD), also referred to as 'glitazone', that belongs to a class of antidiabetic drugs that improves insulin sensitivity. Rosiglitazone is an agonist and acts by activating peroxisome proliferator-activated receptor gamma (PPARγ). Activation of PPARγ alters the transcription of genes involved in glucose and lipid metabolism and the energy balance, including those that code for lipoprotein lipase (LPL), fatty acid transporter protein, adipocyte fatty acid binding protein and fatty acyl-CoA synthase. Rosiglitazone reduce insulin resistance in adipose tissue, muscle and liver. However, PPARγ is predominantly expressed in adipose tissue.(Hauner 2002). Rosiglitazone markedly influence lipid metabolism by decreasing plasma triglyceride, free fatty acid and LDL-cholesterol levels, and by increasing plasma HDL-cholesterol concentration. Even though rosiglitazone do not stimulate insulin secretion, it does improve the secretory response of beta cells to insulin (Komers and Vrana 1998).

1.5.3 Tetradecylthioacetic acid (TTA)

Tetradecylthioacetic acid (TTA) is a non-β-oxidizable fatty acid analogue, which regulates lipid homeostasis. TTA has been shown to both up-and down regulate a number of genes encoding for regulatory factors in lipid metabolism, most likely through the action of lipid-activated transcription factors like PPARs (Hihi, Michalik et al. 2002). TTA activates all three isotype PPAR in the ranking order PPAR alpha > PPAR beta/delta > PPAR gamma. Studies shows that a TTA-induced increase in hepatic fatty acid oxidation, together with ketogenesis draining fatty acids from blood and extrahepatic tissues, may contributes to a beneficial effect on fat mass accumulation and peripheral insulin sensitivity (Madsen, Guerre-Millo et al. 2002).

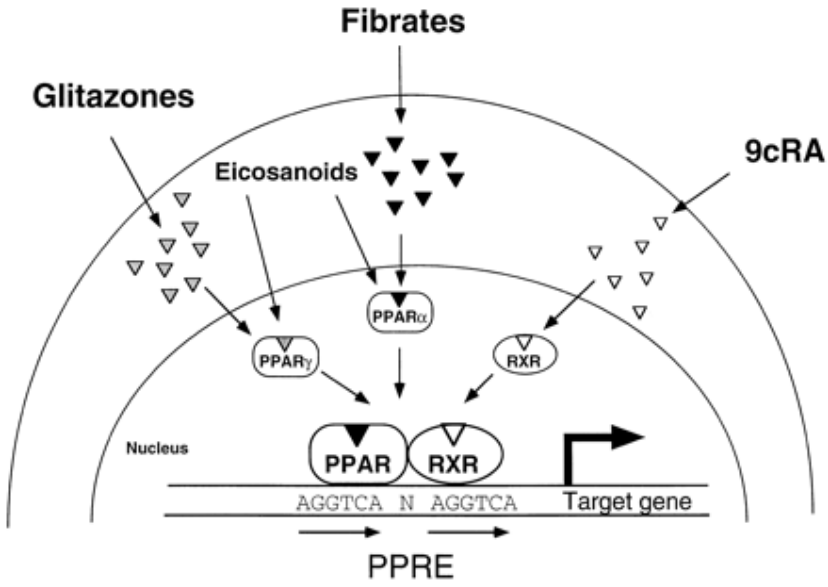

Figure 1.6 The PPAR signaling pathway and its natural and synthetic activators. Activation by its respective ligands, PPARs heterodimerize with the receptor for 9-*cis*-retinoic acid (9cRA), RXR, and bind to specific REs in the regulatory regions of target genes (Staels, Dallongeville et al. 1998).

1.6 PPARs target genes

1.6.1 Scavenger receptor class B type 1 (SR-B1) and CD 36

SR-BI and CD 36 are both members of the scavenger receptor superfamily which contains a vast variety of transmembrane receptors different in structure, expression and function but with the common feature of binding modified LDL. The scavenger receptor superfamily is divided into six classes A-F (figure 1.7) (Greaves, Gough et al. 1998).

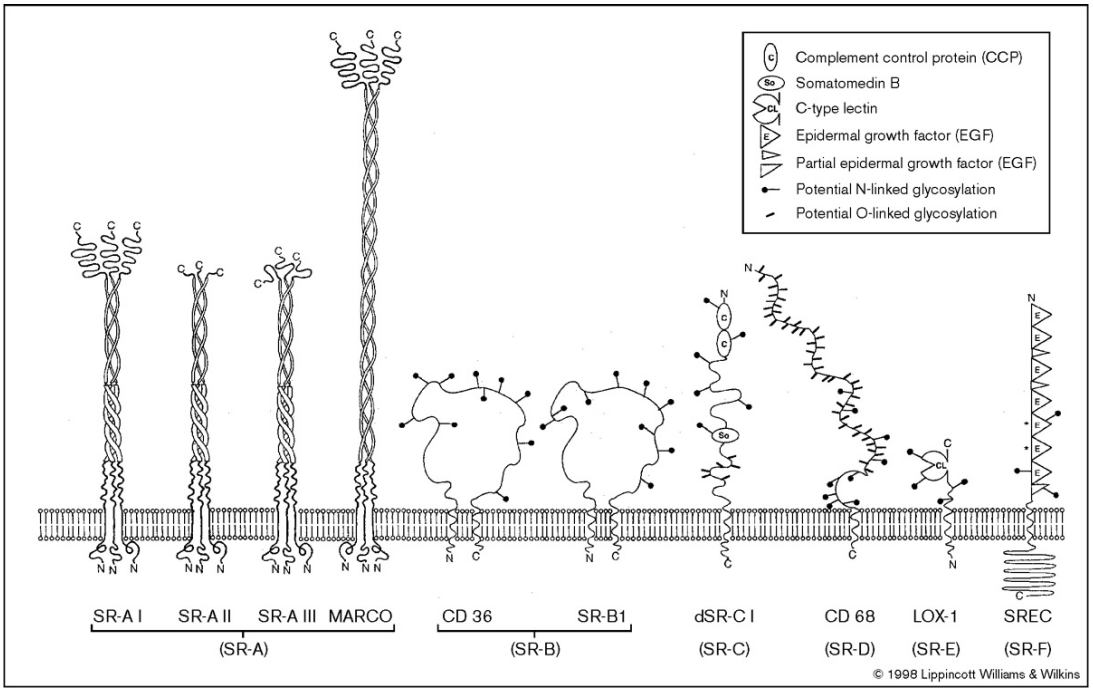

Figure 1.7 The scavenger receptor superfamily, classification and their proposed structure (Greaves, Gough et al. 1998).

SR-BI is mainly expressed in tissues with critical roles in cholesterol metabolism, for instance the liver and steroidogenic tissues. SR-BI is a receptor for native and modified lipoproteins, and mediates the bidirectional exchange of lipids (Acton, Rigotti et al. 1996) SR-BI binds with high affinity, and facilitate both the selective uptake of high density lipoprotein (HDL), cholesteryl ester (CE) and the efflux of cellular cholesterol to circulating HDL particles (Ji, Jian et al. 1997). SR-BI primary function is maintaining cholesterol homeostasis, in addition it has been shown that SR-BI can mediate phagocytosis of apoptotic cells through binding of phosphatidylserine residues on the cell surface (Rigotti, Acton et al. 1995). SR-BI expression level is controlled by hormones, fatty acids, lipoproteins and nutrients through a complex regulatory network of transcription factors (Rhainds and Brissette 2004).

CD 36 is a surface glycoprotein which is expressed by monocyte/macrophages, platelets, microvascular endothelial cells, and adipose tissue (Nicholson, Frieda et al. 1995) CD 36 recognizes a multitude of ligands, and is for instance a receptor for oxidized LDL, long-chain fatty acids and the membrane of cells undergoing apoptosis (Nicholson, Frieda et al. 1995). CD 36 seem to play a strategic role in the lipoprotein and lipid metabolism (Febbraio, Abumrad et al. 1999). The major role of CD 36 in fatty acid uptake and lipid metabolism is seen in CD 36-null mice that generate increased plasma levels of cholesterol, triacylglycerol

and fatty acids (Febbraio, Abumrad et al. 1999). Oxidised LDL stimulate its own uptake by induction of CD 36 gene expression, which must involve activation of transcription factor; PPARγ (Nicholson, Han et al. 2001).

1.6.2 Acyl- Coenzym A oxidase (ACO)

Peroxisomes are single membrane-bound cytoplasmic organelles that participate in a variety of metabolic processes. These organelles are present in virtually all eukaryotic cells and are most abundant in mammalian liver and kidney. The most extensively characterized process performed by peroxisomes is fatty acid β-oxidation, a cyclic pathway involved in the degradation of lipids (Wang, Le Dall et al. 1999). Via β-oxidation, peroxisomes play an essential role in both the metabolism of lipids and also in bile acid synthesis (Aoyama, Tsushima et al. 1994). ACO isozymes catalyze the first rate-limiting steps of the peroxisomal β-oxidation, This enzyme is critical for completion of straight chain fatty acid β-oxidation (Ngo, McKinnon et al. 2003). Induction of ACO occurs at a transcriptional level with the activation of PPARα (Reddy, Goel et al. 1986).

1.6.3 Lipoprotein lipase (LPL)

LPL is the extrahepatic enzyme that is responsible for the hydrolysis of triglycerides in plasma lipoproteins. After synthesis in parenchymal cells, LPL is secreted and travels the interstitial space before they bind to the luminal surface of the vascular endothelial cells. The amount of enzymatically active LPL *in vivo* varies greatly with feeding, fasting, and with a number of pharmacologic intervention (Saxena, Witte et al. 1989). LPL mediates the uptake of fatty acids and can to some extent control lipid storage between liver and extrahepatic tissues. LPL activity gives a strong peripheral accumulation of lipids (Griffin, Butterwith et al. 1987; Andre, Guy et al. 2007).

1.6.4 Delta 5 (Δ5) and delta 6 (Δ6) desaturases

Delta 5 and delta 6 are enzymes involved in the metabolic pathway of the long-chain polyunsaturated fatty acids. In the first and rate limiting step in the biosynthesis of n-6 and n-3

polyunsaturated fatty acids (PUFAs), the microsomal Δ6 desaturates the essential fatty acids linoleic acid (18:2n-6) and α-linolenic acid (18:3n-3). The product of these reaction, γ-linolenic (18:3n-6) and stearidonic acids (18:4n-3), are elongated to dihomo- γ-linolenic acid (20:3n-6) and 20:4n-3, respectively. These fatty acids are then desaturated by Δ5 to generate arachidonic acid (20:4n-6) and 20:5n-3 (figure 1.8). Further elongation and desaturation take place before peroxisomal β-oxidation converts the product to their final state; 22:5n-6 and 22:6n-3 at the end of the pathway (de Antueno, Knickle et al. 2001).

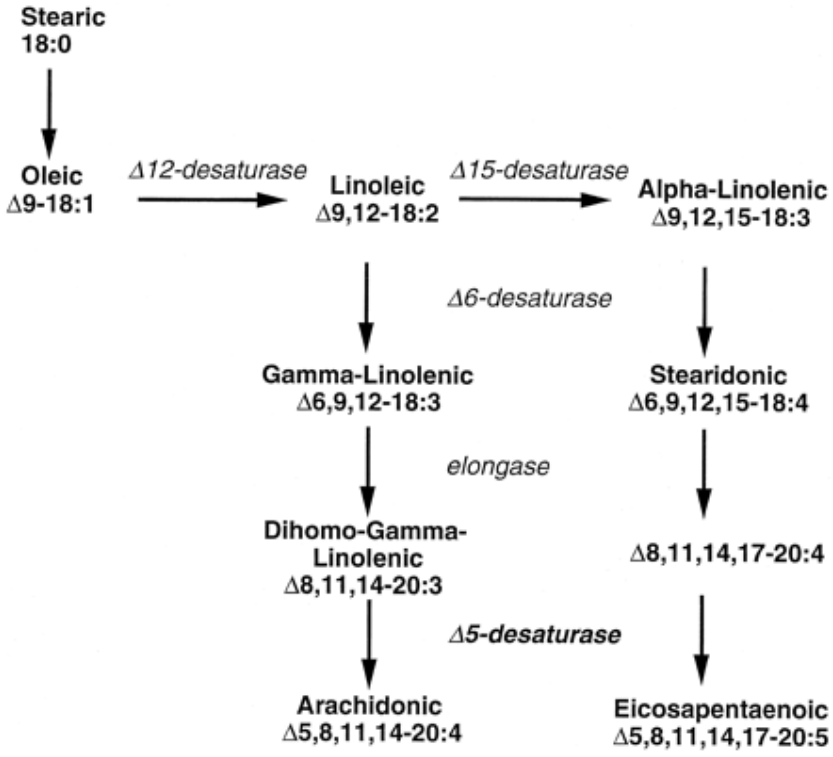

Figure 1.8 Metabolic pathway of long-chain polyunsaturated fatty acids. Further elongation and desaturation from arachidonic acids to β-oxidation step and final products are not shown here. PUFAs, polyunsaturated fatty acids; fatty acids are designated with a number that refers to the number of carbons in the chain; the number after the colon indicates the number of double bonds and the n refers to the location of the nearest double bond from the methyl end (Knutzon, Thurmond et al. 1998).

1.7 Real-Time Polymerase Chain Reaction

1.7.1 Detection of low-abundance mRNA

Real-time reverse transcription polymerase chain reaction (RT-PCR) applying fluorescence dyes (for example SYBR Green I) is currently the most sensitive and precise method for detection of low-abundance mRNA, often obtained from limited tissues samples. This highly sensitive technique has become the method of choice for detecting mRNA (Bustin 2000). RT-PCR method allows measurement of different type RNA level in the cell, based on the kinetics of the corresponding double-stranded cDNA amplification. The SYBR Green I dye binds to the minor groove of double –stranded DNA, and its fluorescence increases about 100-fold. As the fluorescence signal is increasing it is recorded at early cycles of amplification (Nikitina, Nazarova et al. 2003).

1.7.2 The theory behind Real-time PCR

Real-time PCR is the technique of collecting data throughout the PCR reaction as it occurs; in addition carry out amplification and detection in one single step. All together makes it less labor-intensive than other quantitative PCR methods. This is achieved by using different fluorescent chemistries that correlate PCR product concentration to fluorescence intensity. Reactions are characterized by the point in PCR cycle where the target amplification is first detected. This point is usually referred to as the cycle threshold (C_t), the time at which fluorescence intensity is greater than background fluorescence. Consequently, the greater the quantity of target DNA in the starting material, the faster a signal will appear, yielding a lower Ct (Heid, Stevens et al. 1996).

OBJECTIVES

The objectives for this thesis were:

- To study peroxisome proliferator-activated receptor transcription regulation in Atlantic salmon head kidney cells *in vitro*.
- To determine PPARs expression levels in tissues *in vivo*.
- To test specific PPAR agonists and analyze expression of target genes.
- To investigate possible toxic effects of PPAR agonists on Atlantic salmon head kidney cells.
- Perform transfection studies to examine PPARγ.
- To determine if SHK-1 and ASK cells are suitable *in vitro* models for PPARs studies in fish.
- To analyse the specificity of a novel anti - PPARγ antibody.

2 MATERIALS

2.1 Reagents and chemicals

Amersham Biosciences, Buckinghamshire, UK

- ECL plus western blotting detection system
- Hybond^{TM}-P transfer membrane

Applied Biosystems, NJ, USA

- SYBR Green PCR Master Mix

Arcus Produkter, Oslo, Norway

- Alkohol, absolutt Prima (Ethanol)

BDH VWR, England

- Methanol

BD Living Colors

- Monoclonal Antibody (JL-8) Anti-GFP antibody

Bio-Rad, CA, USA

- Laemmli Sample Buffer
- Acrylamid 30% BIS solution

Bio Whittaker, Wokingham, UK

- Trypsin/EDTA
- RPMI 1640 medium

Calbiochem, Ca, USA

- Tween 20 (oilyethylen sorbitan monolaurat)

Electron Microscopy Sciences, Hatfield, PA

- Paraformaldehyde 16 %

Eppendorf, Hamburg, Germany

- Water, Molecular Biology Grade

GIBCO BRL, Uxbridge,UK.

- Foetal bovine serum (FBS)
- Foetal calf serum (FCS)
- Leibovitz's L-15 medium (L-15)
- 2-mercaptoethanol (2-ME)

Invitrogen, Molecular Probes, Oregon, USA

- Alexa Fluor® 488 phalloidin
- Alexa Fluor® 568 phalloidin
- Prolong® Gold antifade reagent with DAPI
- Prolong® Gold antifade reagent

Jackson Immuno Research Laboratories, Pennsylvania, USA

- Cy^{TM2}-conjugate mouse anti rabbit IgG
- Cy^{TM3}-conjugate mouse anti rabbit IgG

Merck, Darmstadt, Germany

- HCl
- KCl
- KH_2PO_4
- NaCl
- $Na_2HPO_4 * 2H_2O$
- Aceton

Normilk AS, Levanger, Norway

- Non-fat skimmed milk

Pierce, USA

- Supersignal® West Femto Maximum sensitivity Substrate

Sigma-Aldrich, St.Louis, USA

- Bovint serum albumine (BSA)
- Ammonium persulfat (APS)
- Dimethylsulfoksid (DMSO)
- Gentamicin Sulfate
- L-Glutamine
- Triton X-100
- (N,N,N,N,-tetramethyl-Ethylendiamin) (TEMED)
- Trypan Blue
- Anti-Actin antibody produced in rabbit

2.2 Kits

Nucleofector® Solution (Amaxa Biosystems, USA)

RNeasy® Mini Kit (Qiagen, MD, USA)

RNase free DNase set (Qiagen, MD, USA)

TaqMan® Reverse Transcription Reagents (Applied Biosystems, NJ, USA)

2.3 Solutions

Table 2.1: Phosphate buffered saline (PBS) pH 7, 4 (4 L)

NaCl	32 g
KCl	0,8 g
KH_2PO_4	1,08 g
$Na_2HPO_4 * 2H_2O$	7,12 g
MQ water	3950 ml
HCl	To adjust pH to 7,4

Table 2.2: Tris/EDTA (TE) pH 7, 6 (20 ml) (to dissolve primer-concentrate)

1 M Tris pH 7,6	0,2 ml
0,5 M EDTA pH 8,0	0,04 ml
MQ water	19,76 ml

Western blotting:

Table 2.3: 10 % gel

MQ water	1,5 ml
Lower gel buffer	0,9 ml
Acrylamid	1,2 ml
10 % APS	18 µl
TEMED	1,8µl

Table 2.4: Stacking gel

MQ water	1,2 ml
Upper gel buffer	0,5 ml
Acrylamid	0,6 ml
10 % APS	10 µl
TEMED	2 µl

Table 2.5: TGS-buffer

TGS x 10 (Bio-Rad)	100 ml
ddH_2O water	900 ml

Table 2.6: TG blotting-buffer

TG x 10 (Bio-Rad)	100 ml
Methanol	200 ml
ddH_2O water	700 ml

Table 2.7: PBS-T (0, 1 %)

PBS	500 ml
Tween 20	500 µl

Table 2.8: Blocking solution (5 %)

PBS	10 ml
Tween 20	10 µl
Non-fat skimmed milk	500 µl

Table 2.9: Stripping-buffer

100 mM β-mercaptoethanol	390 µl
2 % (w/v) Sodium dodecyl sulphate (SDS)	5000 µl
62,5 mM Tris-HCL pH 6,7	3125 µl

2.4 Synthetic PPAR ligands

Hypolipidemic agent

- Fenofibrate

Hypoglycemic agent (thiazolidinediones):

- Rosiglitazone

Fatty acyl-CoA dehydrogenase inhibitors:

- Tetradecylthioacetic acid (TTA)

(All ligands were dissolved and diluted in DMSO to concentration 100 mM)

2.5 Primers used for quantitative Real-time PCR

Target gene	Direction	Sequence	Primer efficiency
18S*	F (5'-3') R (5'-3')	TGTGCCGCTAGAGGTGAAATT GCAAATGCTTTCGCTTTCG	1.00
EF1-α*	F (5'-3') R (5'-3')	CACCACCGGCCATCTGATCTACAA TCAGCAGCCTCCTTCTCGAACTTC	0.97
PPAR-α**	F (5'-3') R (5'-3')	TCCTGGTGGCCTACGGATC CGTTGAATTTCATGGCGAACT	1.99
PPAR-β**	F (5'-3') R (5'-3')	GAGACGGTCAGGGAGCTCAC CCAGCAACCCGTCCTTGTT	2.07
PPAR-γBoth**	F (5'-3') R (5'-3')	CATTGTCAGCCTGTCCAGAC TTGCAGCCCTCACAGACATG	2.04
PPAR-γLong***	F (5'-3') R (5'-3')	CATTGTCAGCCTGTCCAGAC TTGCAGCCCTCACAGACATG	2.0
PPAR-γShort***	F (5'-3') R (5'-3')	ATACAGCGTGTATCAAGACG TTGCAGCCCTCACAGACATG	2.0
SR-BI**	F (5'-3') R (5'-3')	AACTCAGAGAAGAGGCCAAACTTG TGCGGCGGTGATGATG	1.99
LPL**	F (5'-3') R (5'-3')	TGCTGGTAGCGGAGAAAGACAT CTGACCACCAGGAAGACACCAT	2.0
ACO**	F (5'-3') R (5'-3')	CCTTCATTGTACCTCTCCGCA CATTTCAACCTCATCAAAGCCAA	2.03
CD 36***	F (5'-3') R (5'-3')	GGATGAACTCCCTGCATGTGA TGAGGCCAAAGTACTCGTCGA	1.98
Δ-5***	F (5'-3') R (5'-3')	AGAGGCACTCCCACAGAAGC AGACCTTCCTGTCGATGACCA	2.03
Δ-6***	F (5'-3') R (5'-3')	AGAGCGTAGCTGACACAGCG TCCTCGGTTCTCTCTGCTCC	1.98

Primers for quantitative Real-Time PCR used in the present thesis:
*Already designed and tested by S.M Jørgensen (Jørgensen, Kleveland et al., 2006)
**Already designed and tested by E.J Kleveland (Kleveland et al., 2006)
***Designed by T.Gjøen and tested by A.L Rishovd (Department of Pharmaceutical Biosciences, School of Pharmacy, University of Oslo)

2.6 Cells

- SHK-1 cell line (Salmon head kidney cells) was kindly provided by B. Dannevig (National Veterinary Institute, Oslo, Norway)
- ASK cells (Atlantic salmon kidney cells) was kindly provided by B. Krossoy (Department of Fisheries and Marine Biology, University of Bergen, Norway)

2.7 Plasmids and antibodies

Cloned PPARγ-plasmids and salmon anti-PPARγ-antibodies were kindly provided by H. Sundvold (AKVAFORSK, Norwegian University of Life Sciences).

Plasmids:

- H10- sscPPARγ2 (cDNA from pig, served as a positive control)
- H15- salPPARγwt (cDNA from salmon, this is the normal variant)
- H16- salPPARγΔ (cDNA from salmon, a splicingvariant with deletion)

Salmon PPARγ antibodies:

- PPI- preimmunt serum (produced in rabbit, and serve as a negative control)
- SAB-final bleed (produced in rabbit, express target gene PPARγ)

3 METHODES

3.1 Cultivations of cells

SHK-1 cells (Salmon head kidney cells) and ASK cells (Atlantic salmon kidney cells) were cultured in Leibovitz L-15 medium supplemented with 50 µg ml-1 gentamicin, 4 mM L-glutamine, 40 µM β-mercaptoethanol, 5% foetal bovine serum for SHK-1 cells and 10 % foetal bovine serum for ASK cells. Cells were routinely split 1: 2,5 every 7-8 days for SHK-1 cells and once every two weeks for ASK cells, both cell lines were maintained at 20°C.

3.2 SHK-1 cells treated with PPAR ligands

SHK-1 cells ($4 * 10^5$ cells, passages 60-70) were seeded in 25-cm^2 flasks and cultured in L-15 medium with supplements. 100 µM PPAR ligands were added 24 hours after seeding, and the cells were harvested at days 1 and 3 of post-treatment. The cells were washed carefully 3 times with cold PBS before RNA isolation.

3.3 RNA isolation of PPAR ligand treated SHK-1

Total RNA from the SHK-1 cells was isolated using the RNeasy® Mini Kit from Qiagen (MD, USA). An on-column DNase digestion step was included to remove potential DNA contamination. All protocols were according to the manufacturer's instructions.

3.4 cDNA Synthesis

All samples were reverse transcribed using TaqMan® Reverse Transcription Reagents (Applied Biosystems, CA, USA) according to the manufacturer's protocols and with random hexamer primers. cDNA was made from 2 µg total RNA. In the last of the 3 experiments, the analyses were duplicated in the reverse-transcription step; this was done to test experimental variation in the reverse-transcription reaction. (Ståhlberg et al., 2004)

The two-step RT PCR Reaction was performed with an Eppendorf Mastercycler Gradient.

Thermal cycling parameters:

Incubation: 25°C for 10 minutes.

RT: 48°C for 30 minutes.

Reverse transcriptase inactivation: 95°C for 5 minutes.

Cooling: 4°C for some minutes and then stored in -20°C

3.5 Amplification

Real-Time PCR was performed with the ABI Prism® 7000 system and gene-specific primers. A 2 x SYBR® Green PCR Mastermix, 0,4 µM of each primer, and the cDNA template were mixed in 25µl volumes.

A two-step PCR was run for 40 cycles.

PCR program used:

Active1: 50°C for 2 minutes

Active2: 95°C for 10 minutes

Denaturation (40 x): 95°C for 15 seconds

Annealing/extension (40 x): 60°C for 1 minute

A dissociation curve was included in the PCR program to make sure that specific products were obtained in each run

3.6 Data analyses and statistics

Housekeeping genes were chosen based on the experimental findings of Jørgensen *et al.* (2006), where reference genes in Atlantic salmon were validated. By using housekeeping genes for relative expression analysis of Real-Time PCR data it is possible to investigate the effect of different treatments on gene expression. Relative expression of the different genes was determined using the Relative Expression Software Tool (REST) (Pfaffl, Horgan et al. 2002) An analysis of gene expression in SHK-1 cells treated with PPAR ligands was performed, and normalized by the housekeeping genes 18S and EF1-α (Jorgensen, Kleveland et al. 2006) This analysis sets expression level (ratio between target and housekeeping gene) in control group (untreated SHK-1 cells) to 1, and the changes in the test groups (fenofibrate and rosiglitazone) relative to this. Significant difference from expression in the control group was calculated by the Pair Wise Fixed Reallocation Randomization Test© (2000

randomizations) in the software. Mean values were used in REST, n = 3. Probability values (p) of < 0.05 were considered significant.

3.7 Cell transfection

Cells were cultivated to confluence in 175 cm^2 flask. Cell solution was then trypsinized and equally divided in tubes, each with cell density at least 2 x 10^6 cells per nucleofection® sample. Cell culture tubes were centrifuged at 900 x g at room temperature for 10 minutes. Supernatant was discarded completely so that no medium covered the cell pellet. The pellet was resuspended in room temperature Nucleofector® Solution to a final concentraton of 2 x 10^6 cells/100µl. DNA plasmids were added and the sample transferred into an amaxa certified cuvette. The sample was run in a Nucleofector ® using the program T-20. When the programme was finished the sample was removed from the cuvette. RPMI was added to resuspend the cells and it was transferred from the cuvettes back to tube with a specialised plastic pipette provided in the kit. Culture medium was added to the cells, and the cell medium was cultivated in 20°C.

3.8 Immunofluorescence staining

Cells were cultivated on coverslips in culture dish. The medium was removed and the cells washed with cold PBS. The cells were fixed with 4% PFA or 80% MeOH for 10 minutes. Cells were washed 3 times with PBS-T. PFA-fixed cells were incubated with PBS-/0, 1% Triton for 5 minutes while MeOH-fixed cells stayed in PBS. The coverslips were then blocked in 2% PBS/FCS for 45 minutes.

Staining with antibody:
Primary antibody was diluted 1:200 in 2% PBS/FCS. Coverslips were turned with cells side down over 30µl of antibody mixture and stained for at least 1 hour in room temperature. The coverslips were washed with 2% PBS/FCS three times, carefully. Phalloidin stock solution was first diluted 1: 40 with PBS and then 1:50 in 2% PBS/FCS. Secondary antibody was dissolved in 2% PBS/FCS/Phalloidin in a ratio of 1:200. The staining step was repeated with secondary antibody. The coverslips were washed with 2 % PBS/FCS 3 times before they were permanently mounted with a small drop of Fluorsave with or without DAPI on to objectsglas.

3.8 Phalloidin staining

The cells on coverslips were washed twice with PBS, and then fixed with 4% PFA for 10 minutes before repeating the washing procedure twice or more with PBS. The coverslips were extracted with cold acetone (-20°C) for 3-5 minutes and then washed twice or more with PBS. 5µl phalloidin stock solution was dissolved in 200 µl PBS and then 1 %BSA was added to the staining solution. The cells were incubated in the solution for 30 minutes at room temperature in a covered container to avoid evaporation. The coverslips were washed with PBS and then permanently mounted with a drop of Fluorsave without DAPI on to objectsglas.

3.9 SDS-PAGE and Western blotting

SHK-1 cells transfected with cloned PPARγ plasmids and control cells were seeded in culture dish for 6 days. The medium was removed and the cells washed with cold PBS. A solution of Laemmli sample buffer and 2-ME were added to the cell-culture dish. The lysate was collected with a rubber policeman and homogenized with pipette. The samples were heated in 95°C for 5 minutes before they were separated on a 10% SDS-PAGE gel and blotted on to a Hybond-P membrane, using electro blotting. The membranes were then blocked in 5% non-fat skimmed milk-0, 1% PBS-T for 1 hour. The membranes were then incubated with primary antibodies in their suitable dilution ratio for at least 2 hours in room temperature. All membranes were washed 3 times in 0, 1% PBS-T and then incubated with a secondary antibody. All membranes were washed again 3 times in 0, 1% PBS-T. Chemiluminescence was detected using ECL plus and Supersignal® West Femto detection reagents.

3.10 Cell viability assay

Cells were cultured and treated with PPAR ligands as described above. Trypan blue were diluted in PBS in a ratio of 1:1. On days 1, 3 and 5 of post-treatment, the medium was removed and trypan blue/PBS was added to the cells for 5 minutes. After incubation the solution were removed and PBS were added to the culture dish. The cells were examined in fluorescence microscope for cell death.

4 RESULTS

4.1 Relative expression of PPAR genes in Atlantic salmon tissues

In order to find the relative distribution of individual PPARs in tissues of Atlantic salmon, cDNA of liver, gills and head kidney from 9 fish were tested. The expression of liver and head kidney (two relevant tissues) and gills (less relevant) were investigated by performing quantitative Real-Time PCR. This was to quantify of the variation in expression of PPARγ wt and PPARγ with deletion. Nine fish were analysed for PPAR alpha, beta, gamma long (normal variant) and gamma short (splicingvariant with deletion). The expression was normalized by reference genes 18S and EF1-α in liver, gills and head kidney (Jorgensen, Kleveland et al. 2006). REST© analysis of the Real-Time PCR data showed that there was a significantly lower expression of all PPARs in gills compared to liver. The average expression level in head kidney was also apparently lower than in liver, but this difference was not significant (figure 4.1).

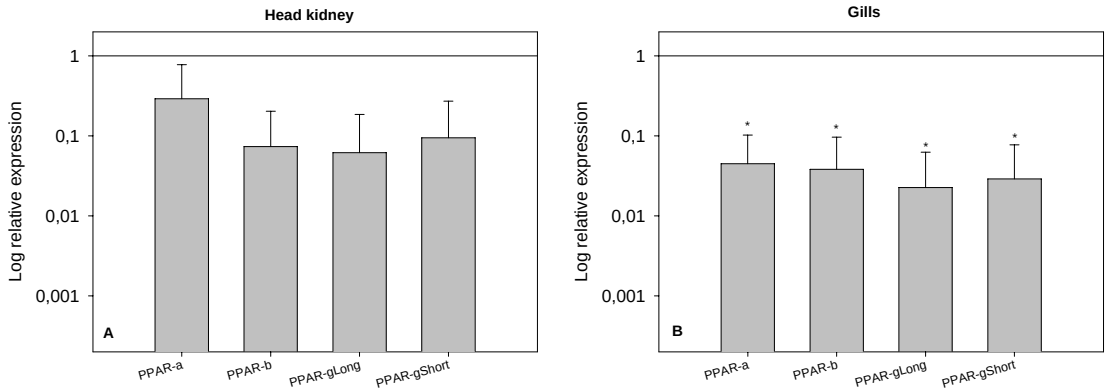

Fig.4.1 Relative expression of PPAR genes in head kidney cells (A) and in gill cells (B) from 9 Atlantic salmon. Relative expression was calculated using the REST© algorithm and the Pair Wise Fixed Reallocation Randomization Test©, (*p*) <0, 05, n = 1. Asterisk (*) above bar denotes significant difference from expression in control cells (liver cells). Abbreviations: PPAR-a, b, g, peroxisome proliferator-activated receptor alpha, beta, gamma.

4.2 Relative expression of PPAR genes in different cell lines

To investigate the expression level of PPARs in different cell lines, and whether they are suitable models for studies of PPAR-regulated genes, we compared the mRNA levels in ASK and SHK-1 muscle and liver tissue samples (liver as normalized samples). PPAR alpha expression was higher in cell lines and muscle. No significant difference was found for PPAR beta, whereas a significantly lower expression was found in for PPAR gamma long and short in all samples compared to liver. Muscle tissue displayed in general the lowest expression (figure 4.2).

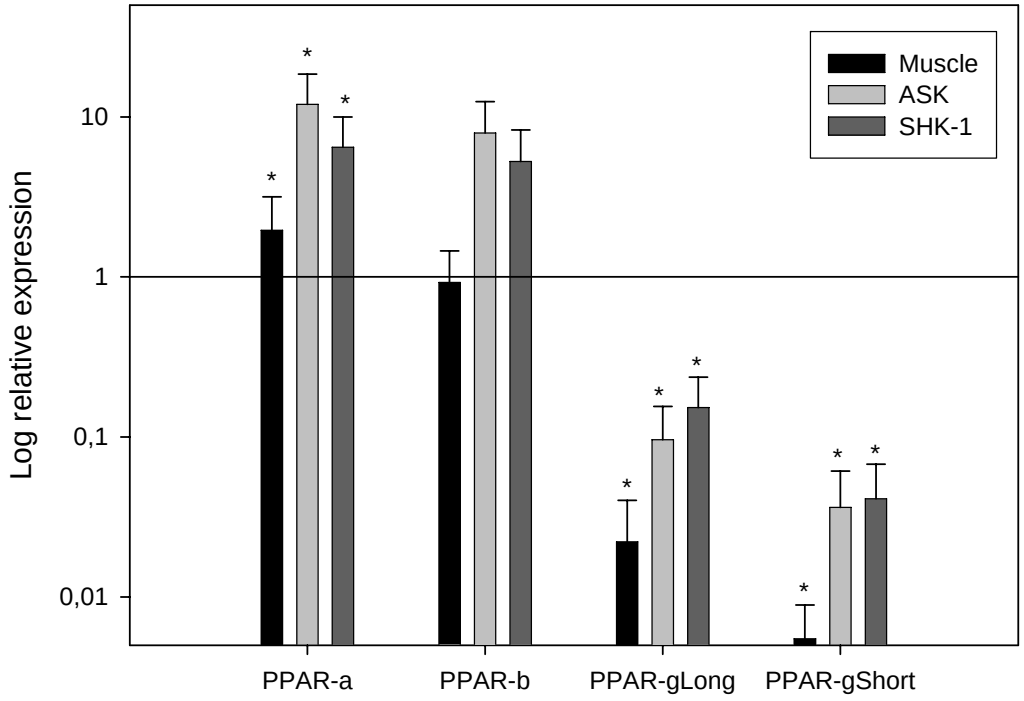

Figure 4.2. Relative expression of PPAR genes in ASK and SHK-1 cells, muscle and liver tissue. Relative expression was calculated using the REST© algorithm. Asterisk (*) above bar denotes significant difference from expression in liver tissue calculated by the Pair Wise Fixed Reallocation Randomization Test©, (*p*) <0, 05, n = 1. Abbreviations: PPAR-a, b, g, peroxisome proliferator-activated receptor alpha, beta, gamma.

4.3 Relative expression of relevant genes in lipid metabolism in different cell

To investigate expression of PPAR target genes in cell lines, we investigated the relative expression of four target genes SR-BI, LPL, ACO and CD 36 (figure 4.3).

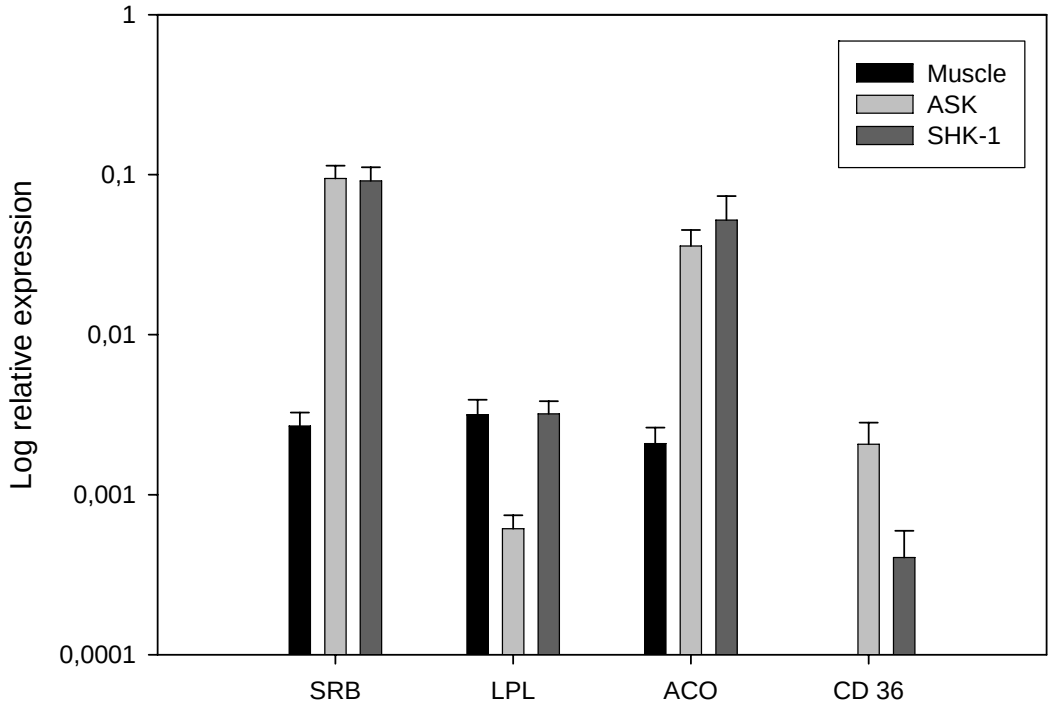

Figure 4.3 Relative expression of target genes in cell tissue and lines; liver, muscle, ASK and SHK-1. Relative expression was calculated using the REST© algorithm and the Pair Wise Fixed Reallocation Randomization Test©, (*p*) <0, 05, n = 2. All the samples are significant down-regulated compared to liver (control sample). Abbreviations; SR-BI, Scavenger Receptor class B; LPL, Lipoprotein Lipase; ACO, Acyl-CoA Oxidase; CD 36, Scavenger Receptor class B submember.

4.4 Comparative pilot of ASK and SHK-1

To investigate possible differences between cell lines, a comparable experiment was conducted with ASK and SHK-1. Both cell lines were seeded and treated with 100 µM TTA for 3 days at 20°C. PPAR genes and target genes in ASK and SHK-1 cells were expressed at comparable levels in both cell lines. The relative expression in both cell lines gave almost the same values, with the exception of LPL, where ASK cells showed a down-regulation whereas SHK did not (figure 4.4).

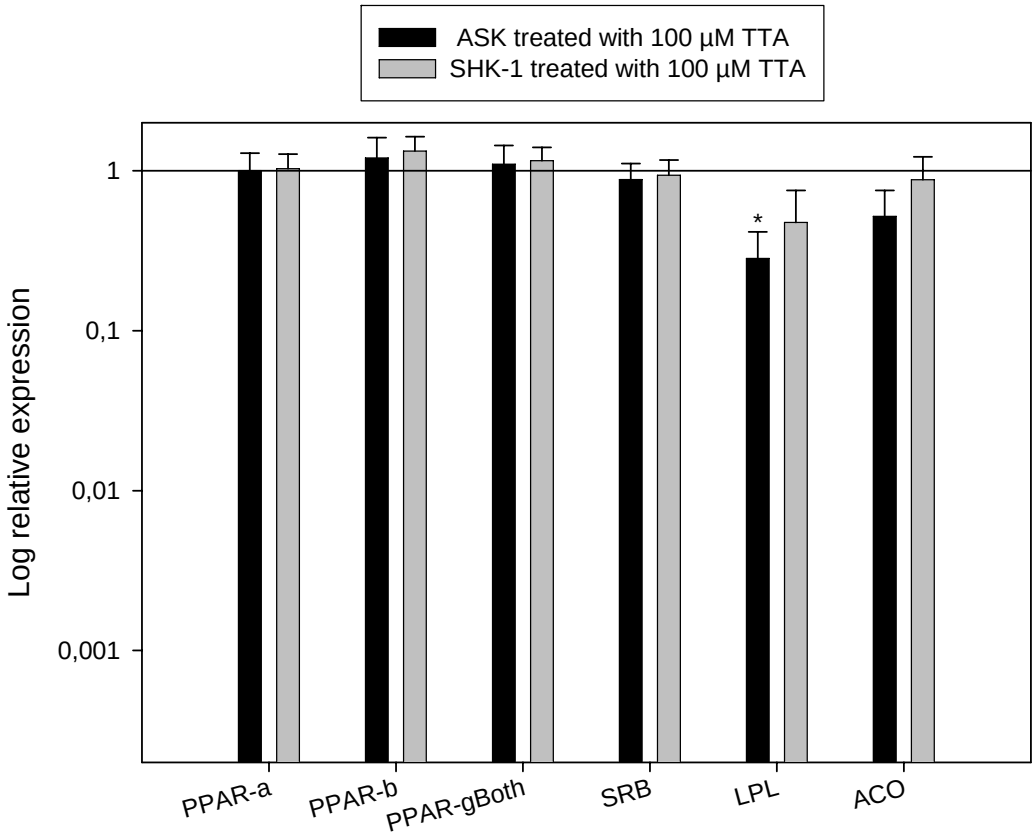

Figure 4.4. Relative expression of PPAR genes and target genes in ASK and SHK-1 cells. Relative expression was calculated using the REST© algorithm. Asterisk (*) above bar denotes significant difference from expression in control cells (un-treated cells) calculated by the Pair Wise Fixed Reallocation Randomization Test©, (*p*) <0, 05, n = 2. Abbreviations: PPAR-a, b, g, peroxisome proliferator-activated receptor alpha, beta, gamma; SR-BI, Scavenger Receptor class B; LPL, Lipoprotein Lipase; ACO, Acyl-CoA Oxidase.

4.5 Morphology changes in cell lines after treatment with PPAR ligands

To see if treatment with 100 μM PPAR ligands could have any toxic effect on the cells, we analysed changes in morphology of both cell lines, ASK and SHK-1. Cells were cultivated on coverslips for 24 hours and then treated with PPAR ligands for 1, 3 and 5 days. After treatment, the cells were formaldehyde-fixed, stained with Alexa-phalloidin and examined for morphology changes by confocal microscopy. ASK cells remained well spread out and unchanged throughout the trial period with fenofibrate and roziglitazone, whereas SHK-1 cells treated with rosiglitazone, showed morphology changes. The cells were changed from a star shaped morphology to more round up and starting to detach from the substrate. Both ASK and SHK-1 cells displayed morphology changes in the presence of 100 μM TTA (figure 4.5 and 4.6). At this concentration, lipid droplets could be observed in the medium and the cells started to detach from the coverslips.

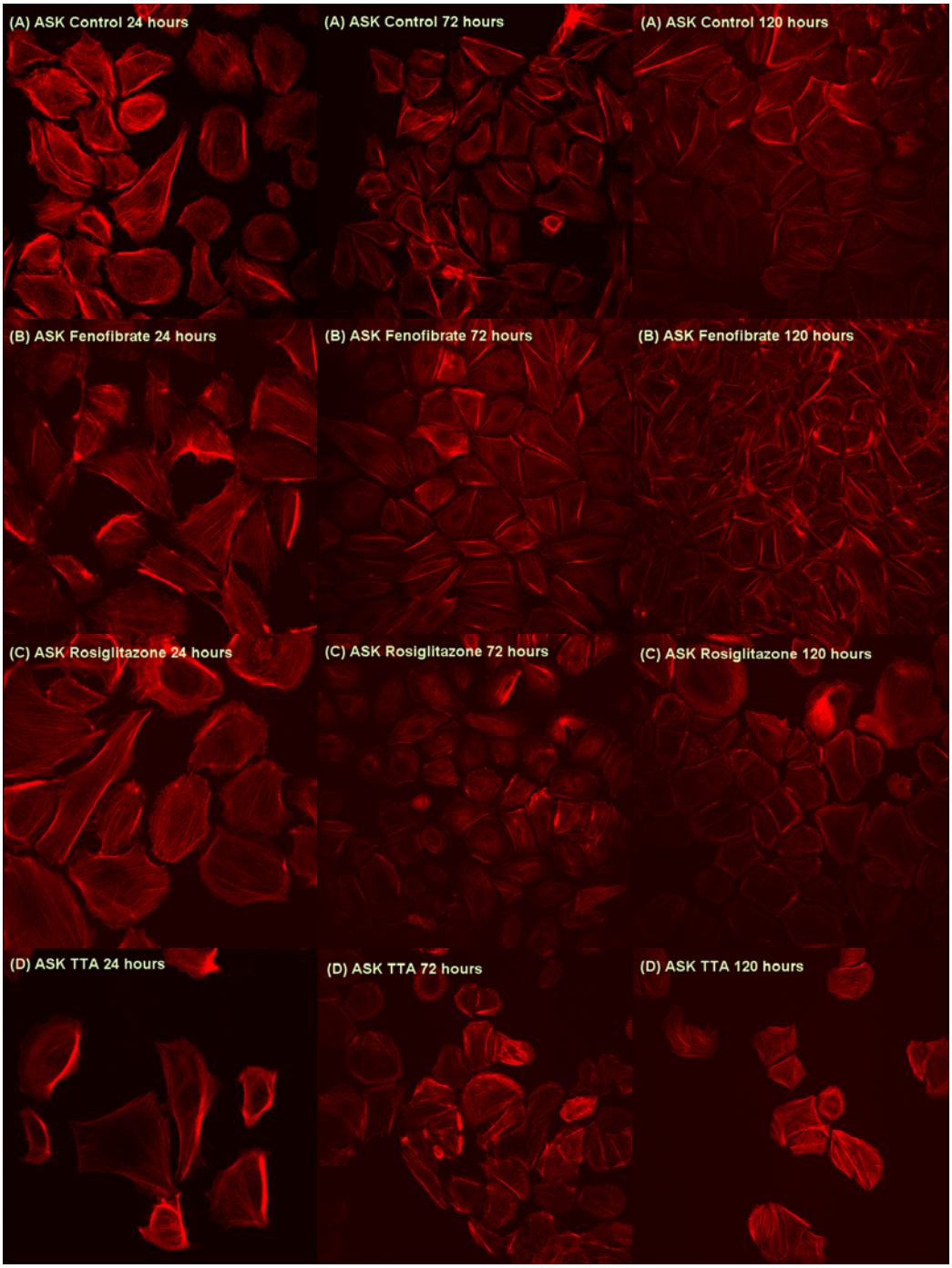

Figure 4.5 Morphology changes in ASK cells. Confocal micrographs of untreated ASK cells (24, 72 and 120 hours) under control culture conditions (A), and following treatment with 100 µM PPAR ligands; Fenofibrate (B), Rosiglitazone (C) and TTA (D). This data is representative of n = 3 separate experiments. Cell passages 60-70. Stained with Alexa Fluor® 488 Phalloidin (red).

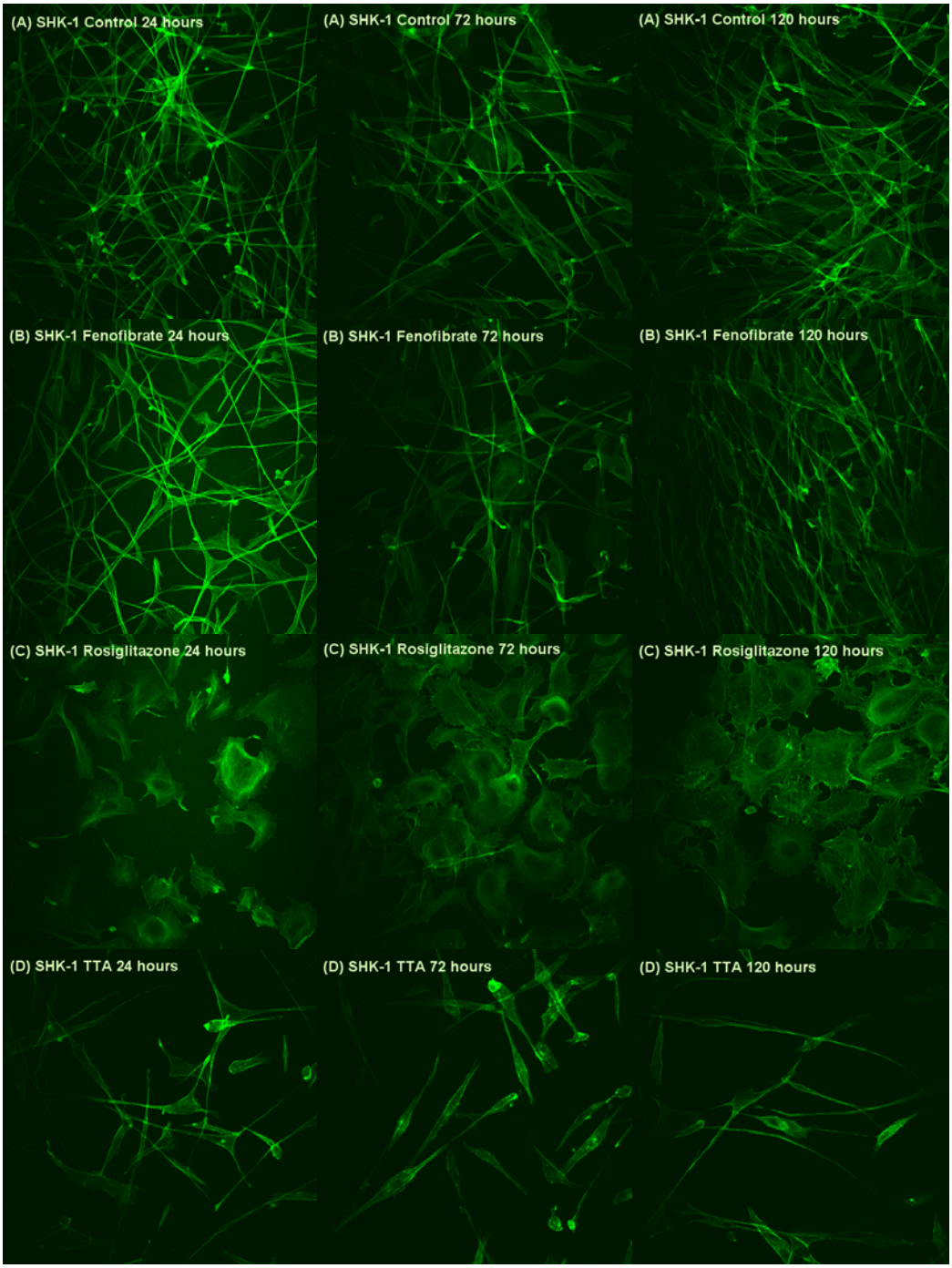

Figure 4.6 Morphology changes in SHK-1 cells. Confocal micrographs of untreated SHK-1 cells (24, 72 and 120 hours) under control culture conditions (A), and following treatment with 100 μM PPAR ligands; Fenofibrate (B), Rosiglitazone (C) and TTA (D). This data is representative of n = 3 separate experiments. Cell passages 75-80. Stained with Alexa Fluor® 568 Phalloidin (green).

4.6 Effect of PPAR ligands on cell viability

To see if treatment with 100 µM PPAR ligands induces cell deaths, ASK and SHK-1 cells were cultivated in culture dish for 24 hours and treated with PPAR ligands for 1, 3 and 5 days. After days 1, 3 and 5 of post-treatment, the cells were stained with trypan blue and examined for dye uptake (cell death) by microscopy (figure 4.7 and 4.8).

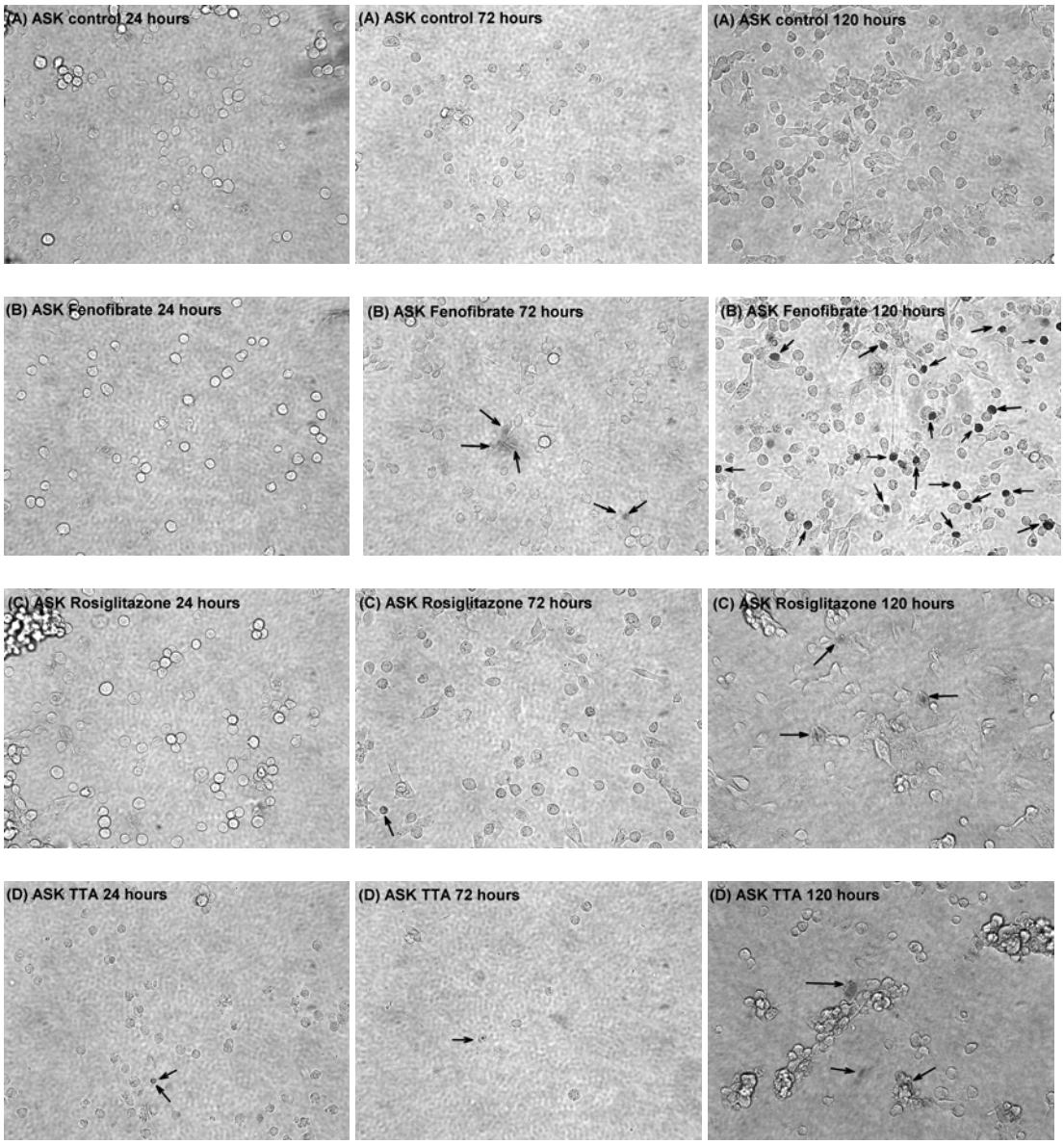

Figure 4.7 Trypan blue staining of ASK cells. Micrographs of untreated ASK cells (1, 3 and 5 days) under control culture conditions (A), and following treatment with 100 µM PPAR ligands; Fenofibrate (B), Rosiglitazone (C) and TTA (D).

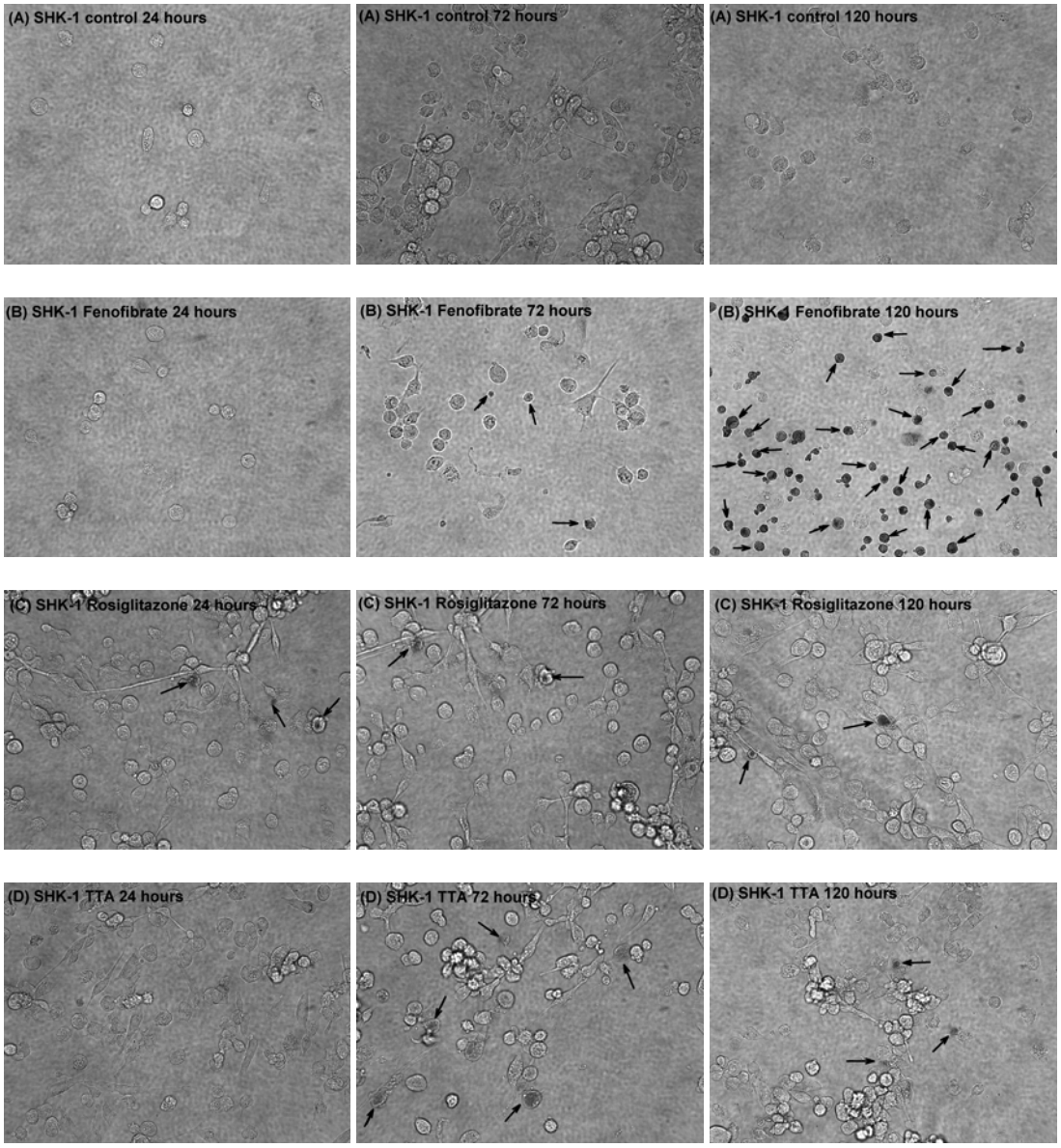

Figure 4.8 Trypan blue staining of SHK-1 cells. Micrographs of untreated ASK cells (1, 3 and 5 days) under control culture conditions (A), and following treatment with 100 μM PPAR ligands; Fenofibrate (B), Rosiglitazone (C) and TTA (D).

Table 4.1: Cell death in ASK cells

Treatment	% cell death 24 hours	% cell death 72 hours	% cell death 120 hours
Control	0	0	0
Fenofibrate	0	5,19	16,98
Rosiglitazone	0	1,23	3,33
TTA	1,63	14,28	3,84

Table 4.2: Cell death in SHK-1 cells

Treatment	% cell death 24 hours	% cell death 72 hours	% cell death 120 hours
Control	0	0	0
Fenofibrate	0	6,81	64,48
Rosiglitazone	3,22	4	4,39
TTA	0	8,45	3,57

4.7 Effect of PPAR agonists on gene expression in SHK-1 cells

To investigate the effects of treatment with PPAR ligands on cells, cDNA of SHK-1 cells treated with 100 μM fenofibrate and rosigliazone for 24 and 72 hours were analyzed with a set of gene specific primers. The mRNA levels of some genes coding for important proteins in lipid metabolism was investigated by quantitative Real-Time PCR. The samples were normalized by the reference genes 18S and EF1-α. In SHK-1 cells treated with fenofibrate for 24 and 72 hours only PPAR gamma short gene at 72 hours showed a down-regulation. SHK-1 cells treated with rosiglitazone for 24 hours showed a significant up-regulation of SR-BI, ACO and CD 36, whereas LPL was down regulated. In cells treated with rosiglitazone a significant up-regulation of SR-BI and CD 36 were observed after 72 h (figure 4.9).

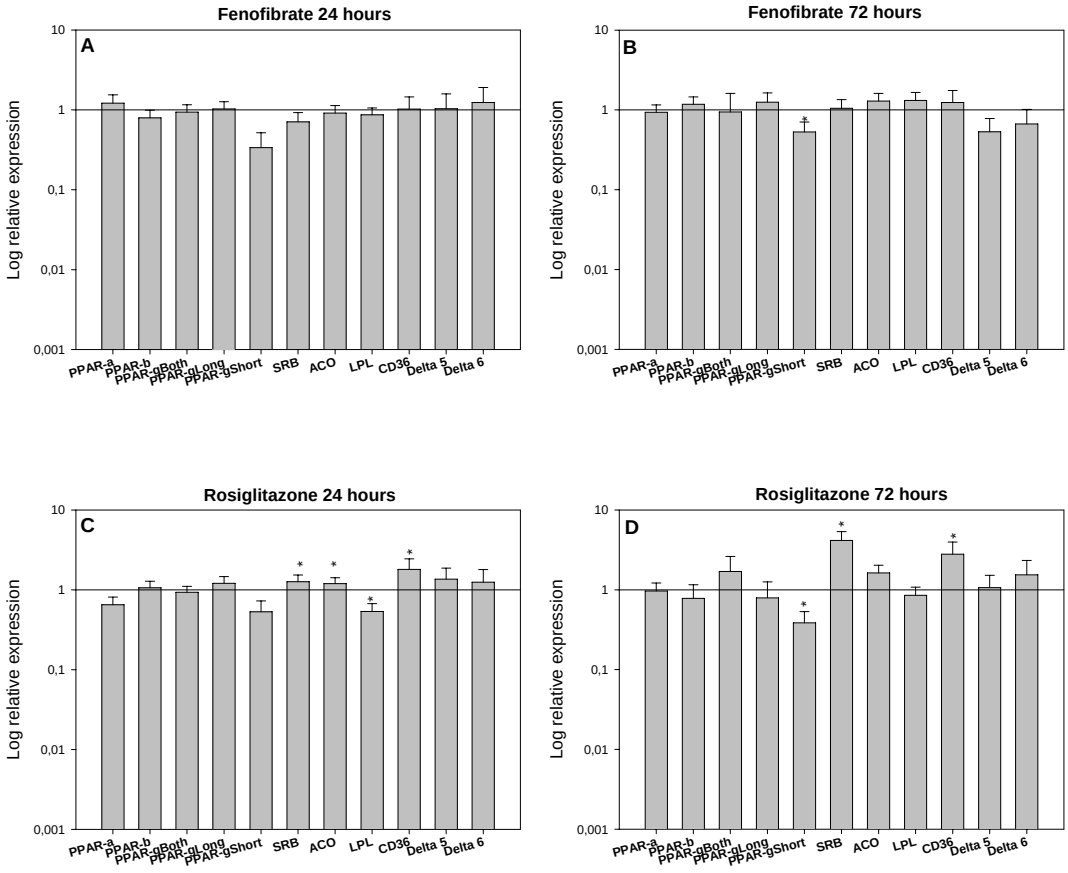

Figure 4.9 Relative expression of genes involved in lipid metabolism in Atlantic salmon head kidney cells (SHK-1) after treatment with PPAR ligands. SHK-1 cells treated with fenofibrate for 24 hours (A) and 72 hours (B), and SHK-1 cells treated with rosiglitazone for 24 hours (C) and 72 hours (D) were maintained at 20°C. Relative expression was calculated using the REST© algorithm. Asterisk (*) above bar denotes significant difference from expression in control cells (untreated SHK-1 cells) calculated by the Pair Wise Fixed Reallocation Randomization Test©, (*p*) <0, 05, n = 3. Abbreviations: PPAR-a,b,g, peroxisome proliferator-activated receptor alpha, beta, gamma; SR-BI, Scavenger Receptor class B; ACO, Acyl-CoA Oxidase; LPL, Lipoprotein Lipase; CD 36, Scavenger Receptor class B antigen; Delta 5,6, Fatty acid desaturase.

4.8 Alignments

Two splice variants of PPARγ have been identified (H.Sundvold unpublished). These cDNAs have been cloned into pCMV-script (expression vector) and called H15 (long) and H16 (short). H16 is the short splicing variant missing 34 aa of A/B domain (coded from exon 3) (see BLAST search figure 4.9). In addition, a PPARγ cDNA (H10) from pig were also available, and served as a control.

BLAST comparison of H15 and H16

```
 Score =  971 bits (2511),  Expect = 0.0
 Identities = 515/549 (93%), Positives = 515/549 (93%), Gaps = 34/549 (6%)

H15  1    VSSYPEMVDTRRAAWSLLSFGLGTLDLVEMDNKMNSFDMKTLSTLDYPYLPSLEYSHNSP
H16  1    VSSYPEMVDTRRAAWSLLSFGLGTLDLVEMDNKMNSFDMKTLSTLDYPYLPSLEYSHNSP

H15  61   HHHHSPDRSHSCNHSPDRSHSFNHSPDRSHSFNHSPDRNHSFNHSPDRSHSFNHSPDRSH
H16  61   HHHHSPDRSHSCNHSPDRSHSFNHSPDRSHSFNHSPDRNHSFNHSPDRSHSFNHSPDRSH

H15  121  SYNDTYSVYQGSVNDKPLSPSQSSDCSIVSLSRPRPHSNPPTYTDASSLLNIDCRVCGDK
H16  121  SYNDTYSVYQ---------------------------------DASSLLNIDCRVCGDK

H15  181  ASGFHYGVHVCEGCKGFFRRTVRLKLVYDHCDLHCRIHKKSRNKCQYCRFQKCLLVGMSH
H16  147  ASGFHYGVHVCEGCKGFFRRTVRLKLVYDHCDLHCRIHKKSRNKCQYCRFQKCLLVGMSH

H15  241  DAIRFGRMPQVEREKLLQAEFMDVEPRNPESADLRALSRQLCLSYHRHFPLTKSKAKAIL
H16  207  DAIRFGRMPQVEREKLLQAEFMDVEPRNPESADLRALSRQLCLSYHRHFPLTKSKAKAIL

H15  301  SGKTHGNSPFVIHDMKSLTAGQYFINCRQLPVLERQRSVLPPEEPAEELELSVFRRIQFR
H16  267  SGKTHGNSPFVIHDMKSLTAGQYFINCRQLPVLERQRSVLPPEEPAEELELSVFRRIQFR

H15  361  SAEAVQEVTEFTKSIPGFTELDMNDQVILLKYGVIEVMTTMLAPLMNKDGTLFAYGQIFM
H16  327  SAEAVQEVTEFTKSIPGFTELDMNDQVILLKYGVIEVMTTMLAPLMNKDGTLFAYGQIFM

H15  421  TREFLKSLRKPFCEMMEPKFEFAAKFNLLELDDSDMALFFAVIILSGDRPGLVNVKPIED
H16  387  TREFLKSLRKPFCEMMEPKFEFAAKFNLLELDDSDMALFFAVIILSGDRPGLVNVKPIED

H15  481  LQETVLQALELQLKTIHPDCPQLFAKLLQKMTDLRQLVANHVRHIHLLKKQELQMCLHPL
H16  447  LQETVLQALELQLKTIHPDCPQLFAKLLQKMTDLRQLVANHVRHIHLLKKQELQMCLHPL

H15  541  LQEIMRDLY  549
H16  507  LQEIMRDLY  515
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Figure 4.10 Alignment. BLAST search shows that the two genes, H15 and H16 are similar with the exception of 34 amino acids (aa) missing at the position 121 aa in H16.

4.9 SHK-1 cell transfection

SHK-1 cells were transfected with pmaxGFP^{TM}, H10, H15 and H16 plasmids. The transfection efficiency observed in SHK-1 cells transfected with pmaxGFP^{TM} plasmid with a fluorescence microscope was estimated to about 2%. The transfected cells were cultivated in 20°C for 1 and 3 days before RNA isolation and cDNA synthesis. Using splice variant specific primers we could demonstrate that the two cDNA's were expressed in the cells after transfection (figure 4.11).

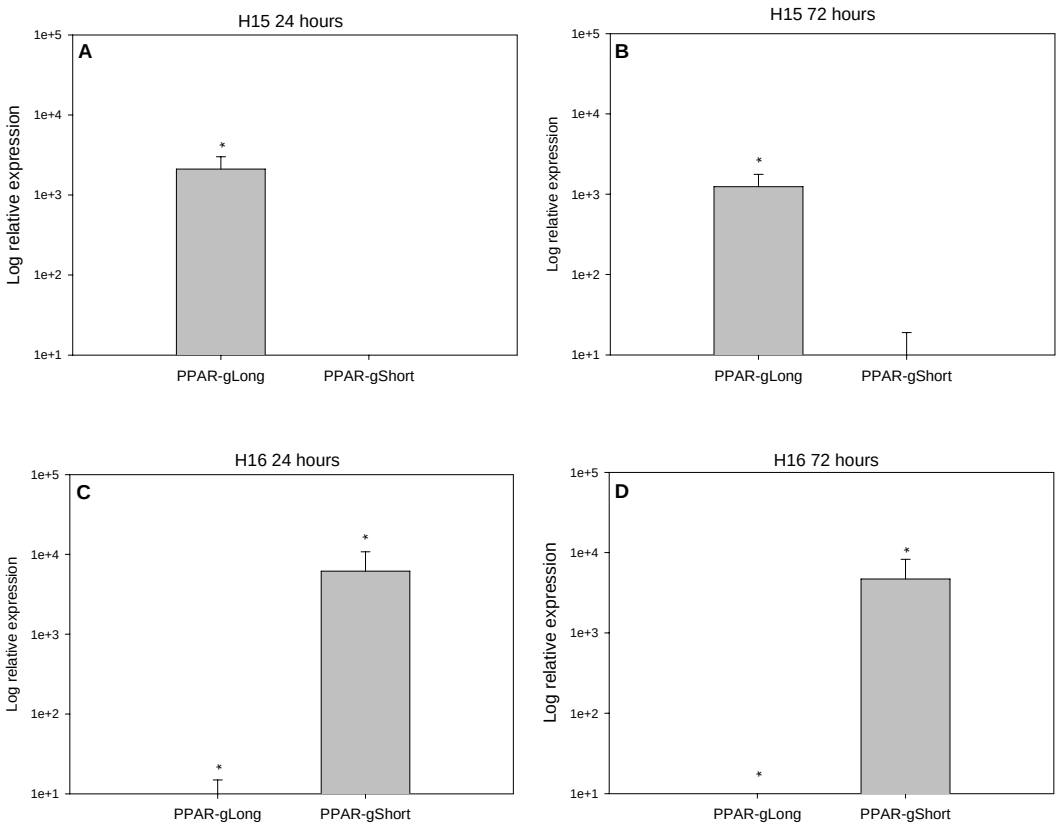


Figure 4.11 Relative expression of PPARγ splice variants in SHK-1 cells transfected with PPARγ plasmids. SHK-1 cells transfected with plasmid H15 (long) for 24 hours (A) and 72 hours (B), and SHK-1 cells transfected with plasmid H16 (short) for 24 hours (C) and 72 hours (D) were all maintained at 20°C. Relative expression was calculated using the REST© algorithm. Asterisk (*) above bar denotes significant difference from expression in control cells (non-transfected SHK-1 cells) calculated by the Pair Wise Fixed Reallocation Randomization Test©, (*p*) <0, 05, n = 2. Abbreviations: PPAR-a, b, g, peroxisome proliferator-activated receptor alpha, beta, gamma.

4.10 SHK-1 cell transfection

Gene expression analysis in SHK-1 cells transfected with H15 and H16 showed an up-regulation of some genes in the 24 hours samples, and a down-regulation in the 72 hours samples. The 24 hours H15 transfected cells showed an up-regulation of SR-BI, ACO and CD 36 genes, while in the 72 hours H15 transfected cells, none of these genes were up-regulated. LPL, Delta 5 and Delta 6 were down-regulated. After 24 hours, H16 transfected cells showed an up-regulation of SR-BI and CD 36. A down-regulation of LPL, ACO, Delta 5 and Delta 6 was seen after 72 hours in H16 transfected cells (figure 4.12).

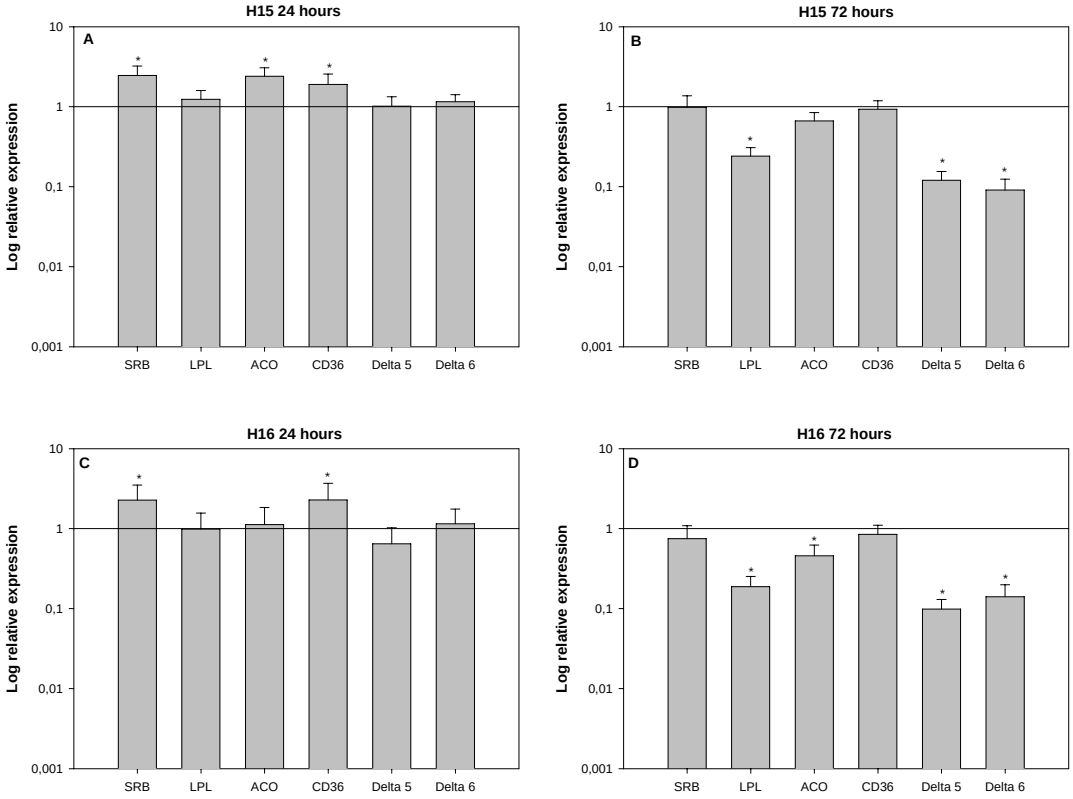


Figure 4.12. Relative expression of some relevant genes in lipid metabolism in SHK-1 cells transfected with cloned PPARy plasmids. SHK-1 cells transfected with plasmid H15 (long) for 24 hours (A) and 72 hours (B), and SHK-1 cells transfected with plasmid H16 (short) for 24 hours (C) and 72 hours (D) were all maintained at 20°C. Relative expression was calculated using the REST© algorithm. Asterisk (*) above bar denotes significant difference from expression in control cells (non-transfected SHK-1 cells) calculated by the Pair Wise Fixed Reallocation Randomization Test©, (*p*) <0, 05, n = 2. Abbreviations: SR-BI, Scavenger Receptor class B; LPL, Lipoprotein Lipase; ACO, Acyl-CoA Oxidase; CD 36, Scavenger Receptor class B member; Delta 5,6, Fatty acid desaturase.

4.11 Analysis of PPARγ expression with peptide antiserum

SHK-1 cells were transfected with PPARγ and pmaxGFP^{TM} cDNA's as described and cultured for 6 days at 20°C. A peptide antibody anti- PPARγ was tested with western blotting to test if PPARγ was increased in the samples transfected with PPARγ plasmids. The samples (pmaxGFP^{TM}, H10, H15, H16 and control) were subjected to SDS-PAGE (10%) in equal amounts (20µg). The membrane was incubated with primary PPARγ antibody in a 1:1000 dilution for 2 hours in room temperature, and secondary antibody mouse anti rabbit HRP was run in a 1:10000 dilution ratio for 1 hour. The antibody detected 2 major bands at 50 and 60 kDa in all the samples, and the control and pmaxGFP^{TM} samples had bands with signal strengths similar to samples transfected with PPARγ plasmid (figure 4.13). Liver protein was also tested with same antibody PPARγ and showed same bands as samples transfected with PPARγ plasmids (figure 4.14). Transfection did therefore not seem to increase a specific PPARγ signal in these samples.

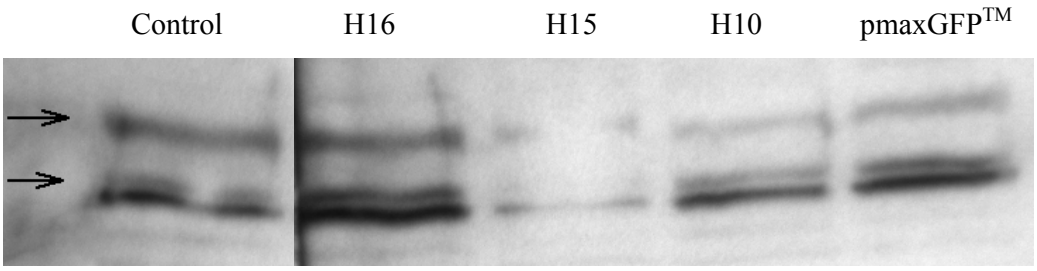


Figure 4.13 Western blot analysis of SHK-1 cells transfected with GFP and PPARy plasmids.
The membrane was incubated with primary anti-PPARy antibody in a 1:1000 dilution for 2 hours in room temperature and with secondary mouse anti-rabbit HRP antibody in a 1:10000 for 1 hour in room temperature. The proteins were visualized with chemiluminescence detection (ECL plus), and the photo negative exposed for 5 seconds. All the samples detected 2 bands at approximately 50- and 60 kDa. The experiment was repeated 3 times.

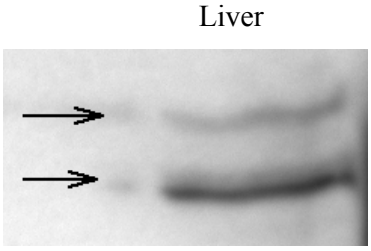


Figure 4.14 Western blot analysis of fish liver proteins. The membrane was incubated with primary anti-PPARy antibody in a 1:1000 dilution for 2 hours in room temperature and with secondary mouse anti-rabbit HRP antibody in a 1:10000 for 1 hour in room temperature. The proteins were visualized with chemiluminescence detection (ECL plus), and the photo negative exposed for 5 seconds. The sample detected 2 bands at approximately 50- and 60 kDa. The experiment was repeated 2 times.

The same samples were tested with anti-GFP antibody. The membrane was incubated with primary anti-GFP antibody in a 1:5000 dilution for 2 hours in room temperature and secondary antibody rabbit anti mouse HRP was run in a 1:50000 dilution ratio for 1 hour. The western blot detected 2 major bands at 27 and 30 kDa in the pmaxGFP^{TM} sample but not in the other samples (figure 4.15).

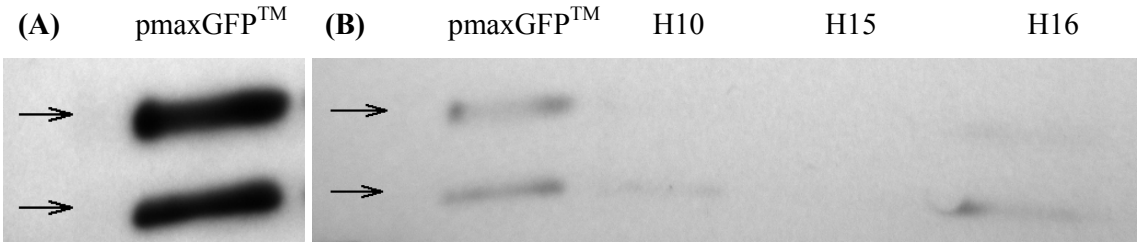


Figure 4.15 Western blot analysis of SHK-1 cells transfected with GFP and PPARy plasmids.
The membrane was incubated with primary anti-GFP antibody in a 1:5000 dilution for 2 hours in room temperature and with secondary rabbit anti-mouse HRP antibody in a 1:50000 for 1 hour in room temperature. The proteins were visualized with chemiluminescence detection (Supersignal® West Femto Maximum sensitivity Substrate), and the photo negative exposed for 5 minutes (A) and 5 seconds (B). Sample (A) is the same as the pmaxGFP^{TM} sample in (B). The pmaxGFP^{TM} sample detected 2 bands at approximately 27- and 30 kDa. The experiment was repeated 3 times.

Sample loading was tested by western blot using an anti-actin antibody. The membrane was run in blocking buffer for an hour in room temperature before incubated with primary anti-Actin antibody in a 1:500 dilution for 2 hours in room temperature, and secondary antibody mouse anti-rabbit HRP were run in a 1:20000 dilution ratio for 1 hour. The western blot detected 1 major band at 42 kDa in all the samples (figure 4.16).

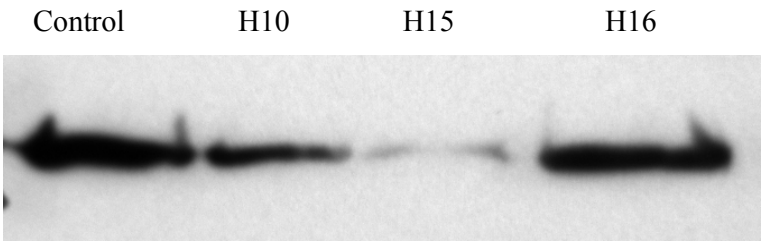
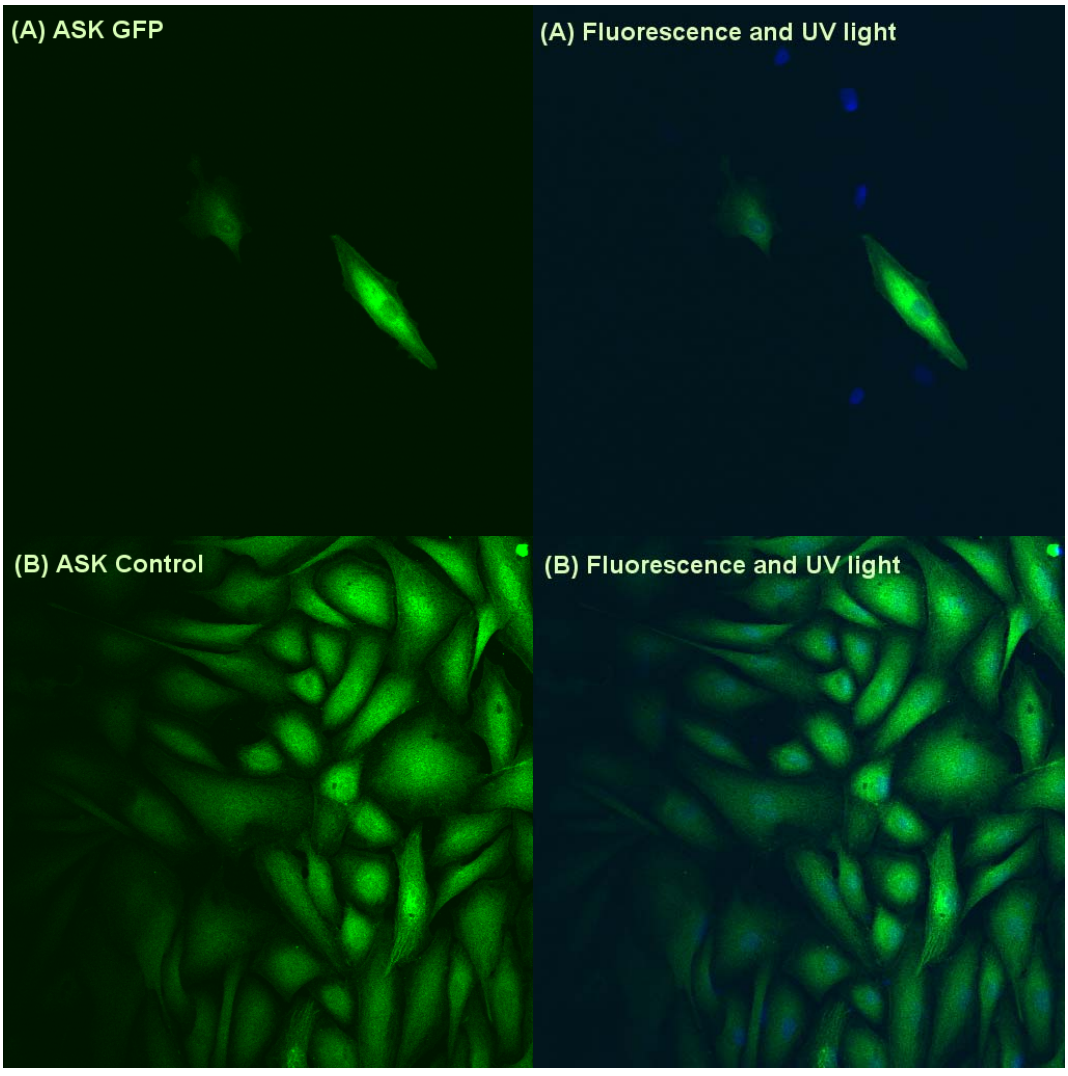


Figure 4.16.Western blot analysis of SHK-1 cells transfected with PPARy plasmids.
The membrane was incubated with primary anti-Actin antibody in a 1:500 dilution for 2 hours in room temperature and with secondary mouse anti-rabbit HRP antibody in a 1:0000 for 1 hour in room temperature. The proteins were visualized with chemiluminescence detection (ECL plus), and the photo negative exposed for 5 seconds. All the samples detected a band at approximately 42 kDa. The experiment was repeated 3 times.

4.12 Immunofluorescence staining of cells transfected with PPARy plasmids

Transfected cells (ASK and SHK-1) were cultured on coverslips for 6 days and stained with PPARγ antibody. The samples were examined in confocal microscope to see if the antibody reacted with transfected PPARγ cells. Cells transfected with pmaxGFP^{TM}-plasmids were included and served as a positive control of transfection efficiency. In the pmaxGFP^{TM} transfected cell samples, only a few (1-2 cells) out of a hundred cells were positive. When cells transfected with PPARγ plasmids and control cells were scanned with the same settings, no differences in fluorescent signal could be observed. Counterstaining with DAPI revealed that all cells were stained, suggesting that the signal either was non-specific, or from endogenous PPAR. There were no differences between the ASK and the SHK-1 cell lines (figure 4.17 and 4.18).

ASK CELLS TRANSFECTED WITH PLASMID (6 DAYS)



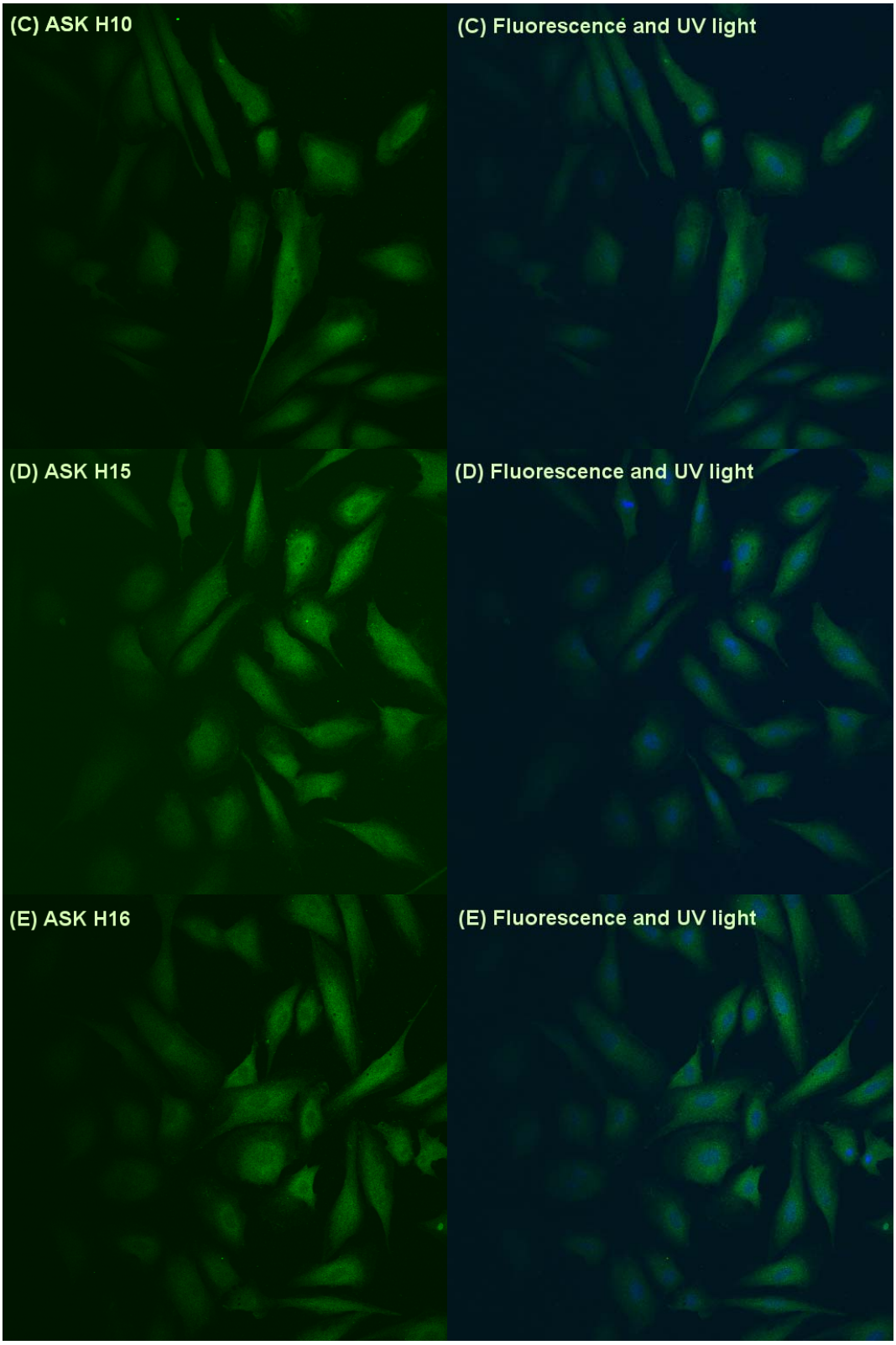


Figure 4.17 PPARy expressions in ASK cells transfected with cloned PPARy plasmids and cultivated for 6 days at 20°C. Immunofluorescence micrographs of pmaxGFP^{TM} transfected cells (A), under control culture conditions (B), transfected with H10 plasmids (C), transfected with H15 plasmids (D) and transfected with H16 plasmids (E).

SHK-1 CELLS TRANSFECTED WITH PLASMID (6 DAYS)

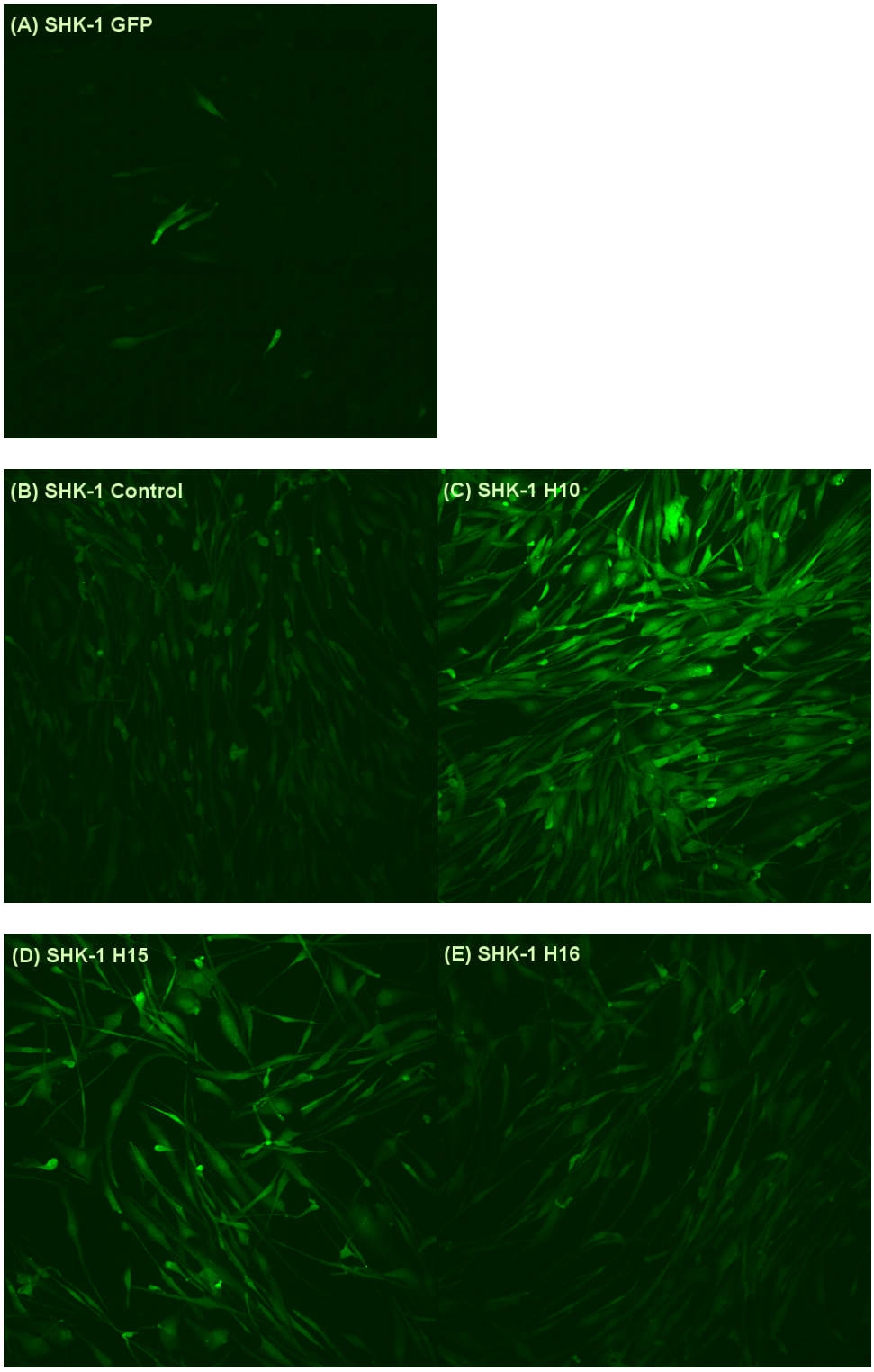


Figure 4.18 PPARγ expressions in SHK-1 cells transfected with PPARγ plasmids and cultivated for 6 days at 20°C. Immunofluorescence micrographs of pmaxGFP^{TM} transfected cells (A), under control culture conditions (B), transfected with H10 plasmids (C), transfected with H15 plasmids(D) and transfected with H16 plasmids(E).

4.13 Immunofluorescence staining of SHK-1 transfected with PPARγ plasmids

Due to low transfection efficiency, transfected SHK-1 cells were cultured and grown on coverslips in culture dish for 3 weeks at 20°C before they were stained with PPARγ antibody and examined in confocal microscope. Although the ratio for transfected cells was higher (approximately 4-5 cells out of a hundred cells) due to longer cultivation time, the results were the same as for transfected cells cultivated for 6 days (figure 4.19).

SHK-1 CELLS TRANSFECTED WITH PLASMID (3 WEEKS)

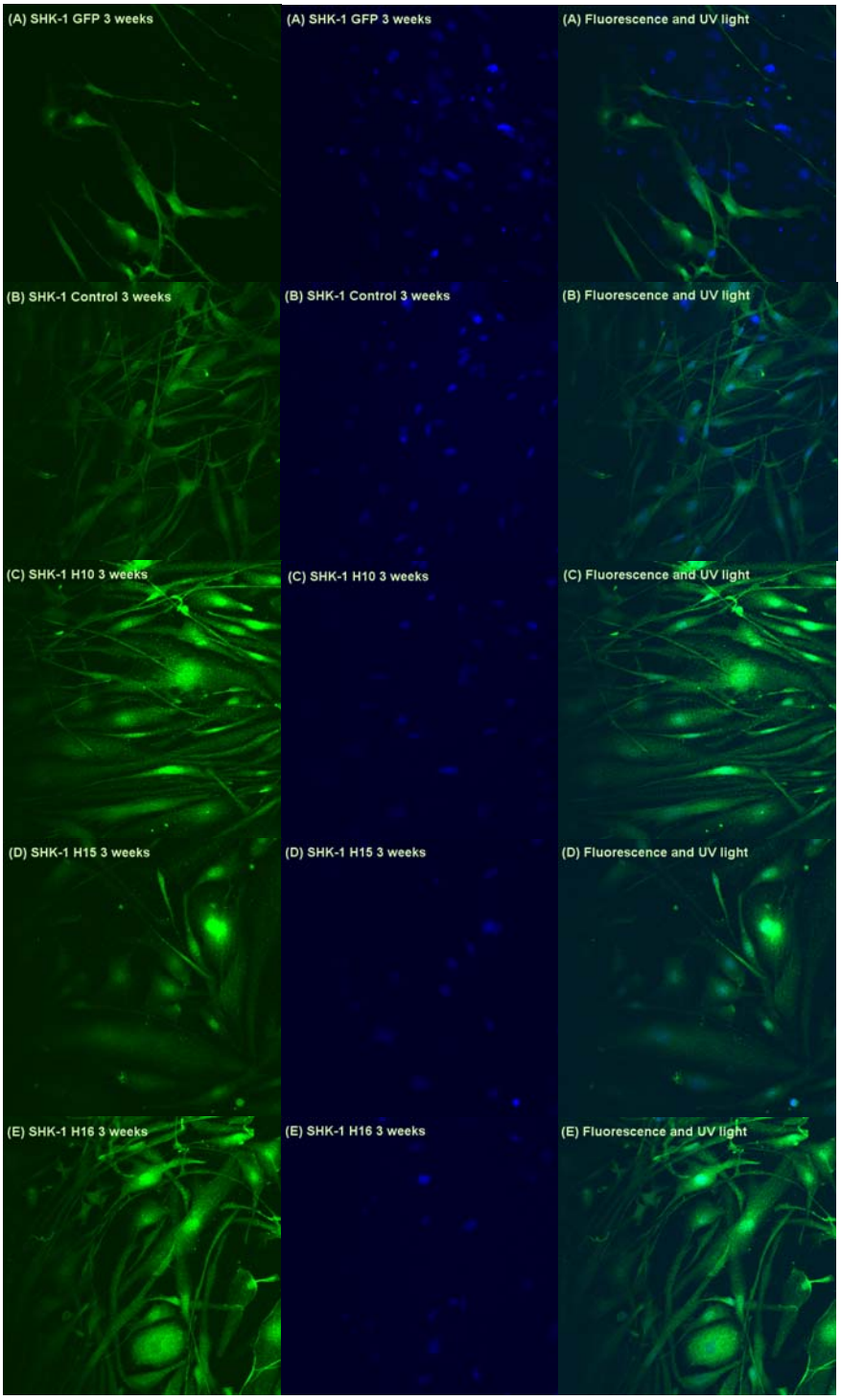


Figure 4.19 PPARγ expressions in SHK-1 cells transfected with PPARγ plasmids and cultivated for 21 days at 20°C. Immunofluorescence micrographs of pmaxGFP^{TM} transfected cells (A), under control culture conditions (B), transfected with H10 plasmids (C), transfected with H15 plasmids (D) and transfected with H16 plasmids (E).

4.14 Immunofluorescence staining of ASK treated with rosiglitazone

In order to investigate whether ASK cells respond to PPARγ agonists by receptor activation and translocation of the receptor from the cytoplasm to nucleus, ASK cells were cultured on coverslips for 24 hours and then treated with 100 μM rosiglitazone for 24 and 72 hours. ASK cells were chosen due to their larger size and flattened morphology. After treatment, the cells were fixed and stained with preimmune serum and PPARγ antiserum (PPI and SAB, respectively) and examined in confocal microscope to analyse for relocalization. One group were fixed with 4% PFA and one with 80% MeOH. There were no observations of relocalization of PPARγ to nucleus in either group and cells treated with primary antibody were all stained throughout the cell (figure 4.20 and 4.21). Figures of ASK cells treated for 24 hours are not shown here. They displayed no difference compared to cells treated for 72 hours.

IMMUNOFLUORESCENCE STAINING 4 % PFA-FIXED

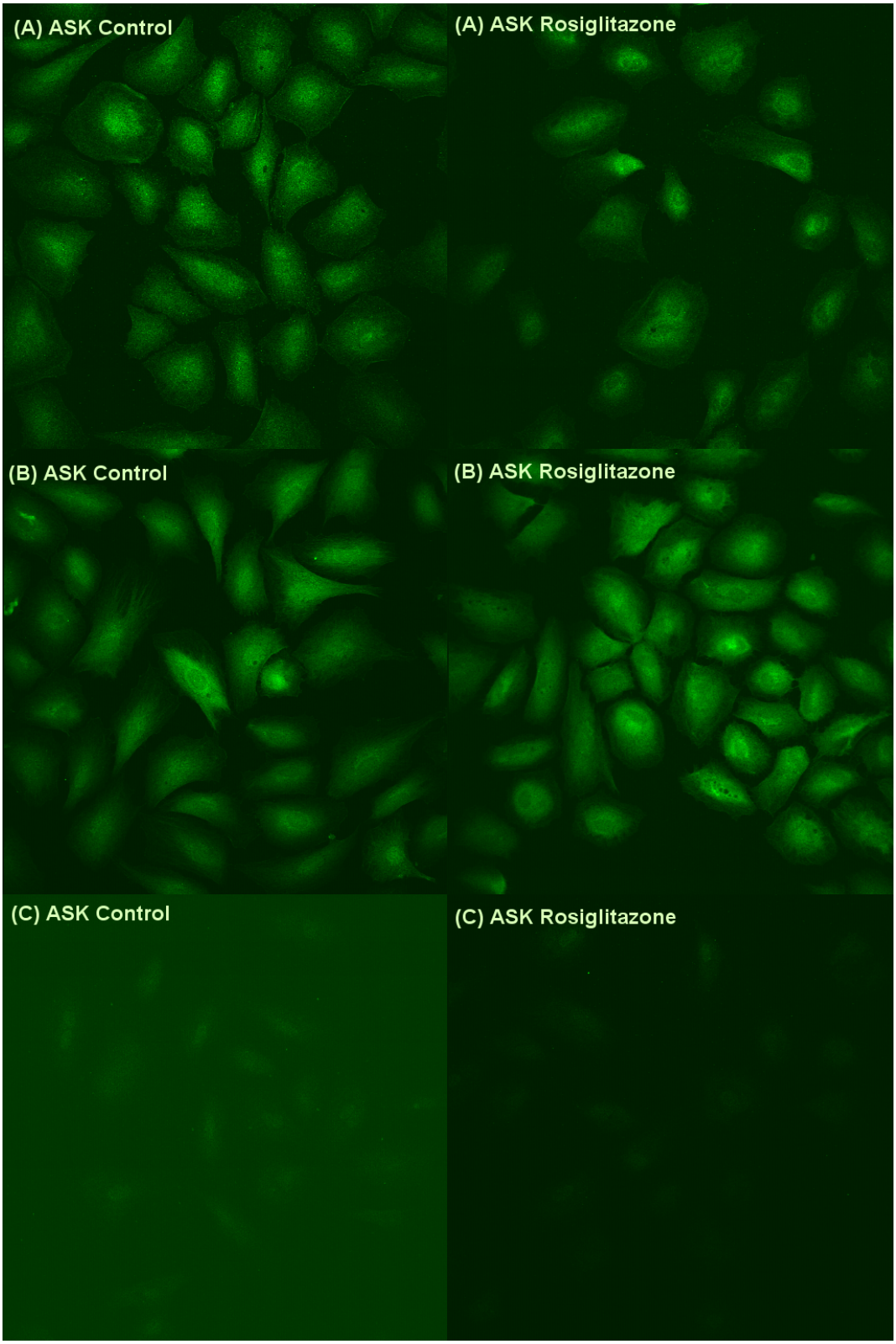


Figure 4.20 ASK cells under control culture conditions and after treatment with rosiglitazone were fixed with 4 % PFA and stained by immunofluorescence. Using preimmune serum (A) anti-PPARy antiserum (B) or only with secondary antibody (C). This data is representative of n = 5 separate experiments.

IMMUNOFLUORESCENSE STAINING 80% MeOH-FIXED

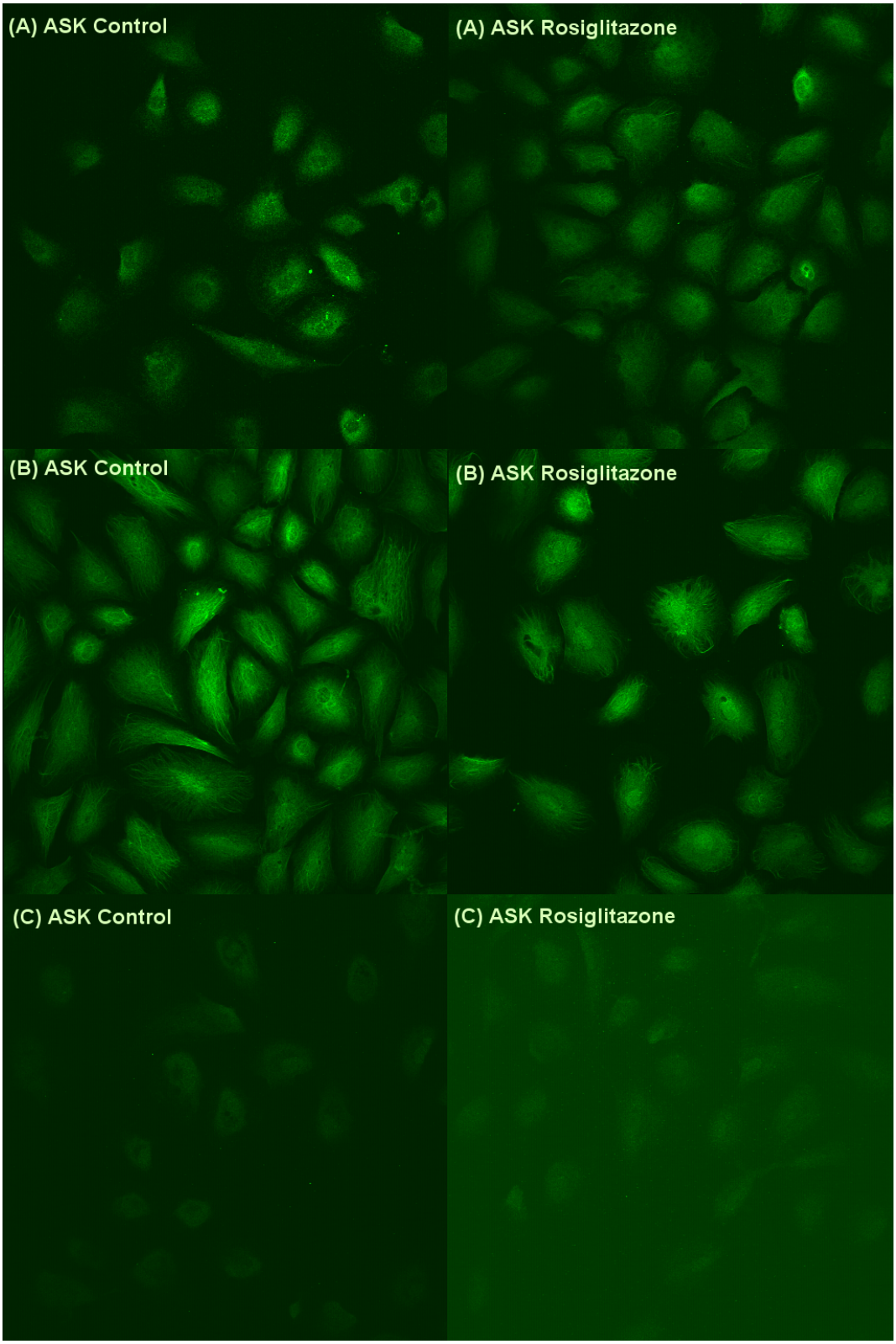


Figure 4.21 ASK cells under control culture conditions and after treatment with rosiglitazone were fixed with 80 % MeOH and stained by immunofluorescence. Using preimmune serum (A) anti-PPARy antiserum (B) or only with secondary antibody (C). This data is representative of n = 5 separate experiments.

5 DISCUSSION

5.1 Methodology

5.1.1 Quantitative Real-time Polymerase chain reaction

The main focus in this thesis was to examine PPAR transcription regulation in Atlantic salmon head kidney cells. Quantitative real-time polymerase chain reaction (qRT-PCR) studies are frequently used for this purpose. Quantitative RT-PCR technology is a more robust and accurate procedure to study gene expression than conventional methods. It is of particular value for expression studies in fish because the availability of highly specific antibodies is still limited. qRT-PCR is now considered as one of the most important methods for rapidly and quantitatively analyzing a number of transcripts from multiple samples in a multiplex format. It is the predominant technique for measuring mRNA levels of low copy number targets of interest. Furthermore, the advantage over conventional methods are a broader dynamic range and high reproducibility (Gibson, Heid et al. 1996; Heid, Stevens et al. 1996). Another major improvement is the ability to normalize data accurately. In order to compensate for errors that may occur during quantification of mRNA transcripts, nonregulated housekeeping genes are amplified simultaneously with target gene to serve as internal reference to which other RNA values can be normalized (Karge, Schaefer et al. 1998). In this thesis 18S and EF1-α were applied as reference gene due to the fact that they are proven to be robust for *in vivo* and *in vitro* normalization of real-time PCR studies in Atlantic salmon specifically (Jorgensen, Kleveland et al. 2006).

5.1.2 Cell transfection

Transfection studies were also performed during this thesis. Transfecting PPARγ plasmid (H15 and H16) into Atlantic salmon cells makes it possible to investigate the transcriptional activity of this particular gene and analyze expression of PPARγ. Transfection studies could reveal important information about the activity in a particular gene. In this case, cDNA construct of two PPARγ splice variants were cloned into pCMV-script (expression vector) and transfected into Atlantic salmon cells with the Nucleofection® method (amaxa biosystems). Most transfection methods are optimized for mammalian cell lines, very few studies are seen using fish cell lines. The experience from previous studies has shown that it is

difficult to obtain high transfection efficiencies in fish compared to mammalian cell line. This was also the case in our experiment. To achieve high transfection efficiency, it is necessary to optimalize the transfection method for fish.

The transfections in this study were performed by the Nucleofector technology method (amaxa biosystems). It is a simple and effective procedure. The principle behind this non-viral method is based on exposure of the cells to a combination of electrical parameters that allows DNA to be rapidly absorbed into the cells. This allows transfected DNA to directly enter the nucleus; it does not rely on cell division as other commonly used non-viral transfection methods. As DNA can directly enter the nucleus, very high transfection efficiency can be obtained in otherwise difficult-to-transfect-cells like immortal culture cell lines. Cell division is not crucial for DNA entry into the nucleus, this makes the time until transgene expression significantly shortened, and the experiment can be performed quickly and within a day.

The nucleofector technology is based on electroporation of the cell and as DNA enters the nucleus directly, expression of transfected protein can be detected shortly after. Although, there are factors that limit the transfection efficiency that should be considered. It is of some importance that the cells are in exponential growth phase when transfections are performed, as this increases the transfection efficiency. The cells undergo mitosis immediately after the transfection and give rapid nuclear entry of DNA into the cells. This can be achieved by keeping the adherent cells subconfluent in culture flask, as this allows one more cell doubling.

The efficiency of transient transfection is also determined by the cell line. Different lines of culture cells vary in their ability to take up and express exogenously added DNA. In our experiment, SHK-1 and ASK cells were used, earlier experiment have shown that Atlantic salmon fish cell line are harder to transfect than most mammalian cells.

All data presented in this study are based on *in vitro* experiments. Transfection is an important tool to study transcriptional activity in a particular gene; however there are limitation related to transfection as a method. Transfection studies alone will not give information of mRNA, protein expression in distinct tissues or how a protein is regulated upon physiological conditions, when compared to *in vivo* studies. A complete picture can only be obtained when *in vivo* and *in vitro* studies are combined.

5.1.3 Cell culture

The main difference between using cultured cells grown *in vitro* (SHK-1 and ASK cells) and the use of an intact animal or an isolated preparation, is that cultured cells provide a more controlled and easily manipulated environment than what is possible to achieve with an intact animal. With cultured cells, there are fewer problems concerning access to the cells of interest and distribution of pharmacological agents. Removing worries like whether or not the liver is metabolizing the substance of interest, makes it possible to control the concentration of an exogenous substance in the vicinity of the cells of interest. It is also possible to selectively alter a single experimental variable and evaluate its effect on a cell with much less concern about possible indirect effect from other systems.

SHK-1 and ASK cells are immortal secondary cell lines. They can be grown and propagated *in vitro* for many years, and the advantage is that they provide a more homogeneous source of cell material than primary cultures, which are cells harvested from a subject, dissociated, and plated into a culture dish where they do not survive for a very long time. In addition, secondary clonal cell lines can be grown in essentially unlimited quantities, which is advantageous when there is need for large amount of cells for biochemical analyses and transfection procedures. However, the fact that the cells can grow indefinitely, implies that they have lost some of the differentiated properties of normal cells, and are acting like cancer cells. In addition, cells that replicate numerous of time are prone to mutations. This must be taken into account and caution must be used when applying the collected information back to cells of intact organism.

In our experiment concerning cell morphology, changes were observed in SHK-1 cells treated with rosiglitazone. The cell passage had exceeded 80, a high passage number for this cell line, and the conformation changes observed could be a result of this, and not by ligand treatment alone.

5.1.4 Trypan blue exclusion viability test

Trypan blue cell stain was observed after incubation of cells treated with ligand for different exposure time. This was particularly evident in cells treated with fenofibrate for 120 hours (5 days). Trypan blue is a non-invasive stain used to selectively colour dead tissues and cells blue. Live cells with intact membranes will not be coloured since the reactivity of trypan blue is based on the chromopore being negatively charged, there will be no interaction unless the membrane is damaged. Therefore, only cells that exclude the dye are viable (Freshney, R,.1987).

The incubation of trypan blue for 5 minutes appeared to have no significant effect on control cells. All cells in control cultures were viable, showing that trypan blue at this concentration do not have a toxic effect on SHK-1 and ASK cells. This indicates that the fenofibrate concentration used in this study was toxic to fish cell lines. Other studies have shown that fenofibrate in higher concentration than 50 μM induces cell cycle arrest and apoptosis in Ishikawa cells (Saidi, Holland et al. 2006). The cell line Ishikawa is established from an endometrial adenocarcinoma. Although different in many aspects, the Atlantic salmon cell lines behave like cancer cells, and it is tempting to hypothesize that fenofibrate induce cell death in ASK and SHK-1 cells at twice as high concentration.

If the PPAR agonist concentration employed here are toxic towards SHK-1 and ASK cells, dead cells would be prone to detach and be washed away during preparation. Only stained cells that still stay onto the surface will be accounted for. With this in mind, the results probably underestimate the number of cell deaths. Using a method with a hemocytometer may be a solution to this problem.

Before staining, the cells were cultered and grown to 100% confluence. After removing medium and staining with trypan blue, the cell confluence was decreased to 30-40 %. The staining procedure itself therefore seems to induce cell detachment from the substrate creating an underestimation of dead cells in the culture.

5.1.5 Protein detection

The use of Western blotting is of great value in immunodetection of proteins. Western blotting, also called immunoblotting, is a powerful procedure for detection of proteins post-electrophoresis, in particularly in our case when the abundance of fish proteins is low. The usefulness of these methods relies on the ability to provide simultaneous resolution of multiple immunogenic antigens within sample, which is different from the majority of immunochemical methods.

Western blotting is effective only when an appropriate detection method is applied. Chemiluminescence method provides the advantageous possibility of re-exposing the blots to obtain suitable intensity for purposes of comparison (Kurien and Scofield 2006). When correctly applied, western blotting is a reliable method for protein detection.

5.2 GENERAL DISCUSSION OF THE RESULTS

PPARs and their ligands have since their discovery kept on fascinating due to their regulatory mode of action of lipids as direct modulators of gene expression. Even though PPARs are one of the most studied nuclear receptors, the knowledge about these receptors is still limited in fish.

Compelling evidence that PPARα and PPARγ act at crucial nodes of the regulatory network to achieve energy homeostasis in the organism, and that lipid mediators like eicosanoids are natural PPAR ligands, give rise to the belief that many aspects of PPAR action are yet to be uncovered.

The activation of PPARs can occur through a broad spectrum of ligands with rather low affinity. This implies that care must be taken when assessing the PPAR dependence of given signalling pathway.

5.2.1 Tissue expression of PPARs in Atlantic salmon

The analysis of PPAR mRNA expression in head kidney and gills compared to liver showed that of PPAR mRNA levels were lower in in head kidney cells, but higher than the level observed in gills. This is consistent with the high levels of PPARα mRNA in liver and kidney, and PPARγ mRNA in adipose tissues in adult rodents (Lemberger, Braissant et al. 1996).

5.2.2 Comparative levels of PPAR in tissues and cell lines

Comparing levels of PPAR mRNA in muscle tissues, ASK and SHK-1 cell lines to liver tissues, revealed that PPARα expression was higher in muscle and cell lines than inn liver. This corresponds to data available in humans were PPARa is well expressed in kidney and muscle amongst other organs (Mukherjee, Jow et al. 1997). Regardless of the species, the expression of PPARα correlates with high mitochondrial and peroxisomal β-oxidation activities, as seen by its high levels in cells of the kidney proximal tubules, which primarily use fatty acids as an energy source. The PPARγ long and short were as expected significant lower in muscle tissue, ASK- and SHK-1 cell lines compared to liver. In adult rodents this is also the case, PPARγ has a restricted pattern of expression with adipose tissues being the major site (Tontonoz, Hu et al. 1994).

5.2.3 PPAR agonists

The concentrations of ligand supplied to culture cells in this thesis were twice as high as standard levels used in similar studies with mammalian cell lines (Saidi, Holland et al. 2006). The concentration was increased because fish cells grown at lower temperature (20°C) have a metabolism that are reduced compared to mammals, and an increase in concentration is often necessary to induce activity. All ligands were added in same concentration for the sake of comparison. One of the challenges in treating cells with ligand solutions is to avoid cytotoxity. Whether the toxicity is time- or dose dependent must be taken into consideration when utilizing these ligands in the investigation of transcriptional regulation in cells.

5.2.4 Effect of PPAR agonists on cell morphology

To investigate whether PPAR agonists had an effect on cells morphology, SHK-1 and ASK cells were cultured and supplemented with PPAR agonist in order to observe changes in a microscope. Neither SHK-1 and ASK cells seemed to change compared to control cells, when treated with fenofibrate for 24, 72 and 120 hours. ASK cells were not affected by treatment with rosiglitazone, whereas SHK-1 showed a significant change in morphology. The mechanism behind this is unknown. Treatment with TTA seemed to have a toxic effect on both cell lines. It has been hypothesized that TTA metabolites in Atlantic salmon accumulate in the kidney, which results in poor growth, inhibition of inflammatory mediator production and thereby increasing mortality (Kleveland, Ruyter et al. 2006). The mechanism behind the findings of Kleveland *et al.* (2006) may be relevant to the observations of TTA induced toxicity in this present study.

5.2.5 Fenofibrate- a PPARα agonist

Fibrates have been used as cholesterol-lowering drugs for many years. Treatment with these agents results in a marked reduction in triglyceride-rich lipoprotein concentrations, a moderate decrease in LDL cholesterol and an increase in HDL cholesterol (Staels, Dallongeville et al. 1998). When 100 μM fenofibrate was supplied to SHK-1 cells, no significant up- or down regulation of either PPAR isotypes or their target genes were observed. The morphology experiment as described earlier, did not show any change in conformation. Although when looking at the results from viability test of both ASK and SHK-1 cell lines, fenofibrate seems to have a toxic effect on the cells at this concentration, this is in agreement with studies on endometrial cancer cells where doses above 50 μM induces apoptosis (Saidi, Holland et al. 2006).

5.2.6 Rosiglitazone- a PPARγ agonist

The present qRT-PCR data show that PPARγ-agonist, rosiglitazone, a thiazolidinedione, stimulates SR-BI and CD 36 expression in SHK-1 cells. These observations are in agreement with the fact that both genes are under the positive control of PPARγ which itself is up-regulated by oxidized LDL (von Schacky, Kiefl et al. 1993). Although viability tests showed no significant increase in cell death, rosiglitazone seemed to have a potent effect on SHK-1 cell morphology. In the study by Saidi *et al*. (2006), it was reported that fenofibrate inhibits

cell proliferation in cancer cells, but the same effect was not seen with high doses of rosiglitazone. This correlates well with our findings.

5.2.7 Expressing PPARγ splicing variants in Atlantic salmon cells

To further investigate the effect of PPARγ in SHK-1, we transfected two PPARγ plasmid variants into SHK-1 cells, H15 (normal variant) and H16 (the short splicing variant missing 34 aa of A/B domain). Cells transfected with H15 gave a significant up-regulation of PPARγ wt variant, and cells transfected with H16 gave a significant up-regulation of PPAR short splicing variant compared to nontransfected cells. Low transfection efficiency (2 %) raise questions about the validity of these results. Although it is unlikely that such low transfection efficiency would lead to the observed up-regulation, a possible explanation could be a self up-regulation by internal activation as a response to increased PPARγ levels.

Applying specific primers on samples transfected with PPARγ, shows up-regulation in SR-BI and CD 36, this is in agreement with PPARγ properties and our earlier findings. It is well established that lipid components of modified LDL activates PPARγ and thereby induced scavenger receptor CD 36 expression in macrophages (Nagy, Tontonoz et al. 1998)

Transfected cells were immunofluoresecence stained in order to detect PPARγ expression. All the samples displayed green fluorescence signal throughout the whole cell. No specific green fluorescence stain could be attributed to PPARγ. Due to the low transfection efficiency (< 2%), it is reasonable to assume that the chosen method and cell line were not optimal for this kinds of study. The low expression of PPARγ in SHK-1 cells and ASK, and the low transfection ratio makes it apparent that a more optimalized procedure is necessary in order study PPARγ expression and activation in Atlantic salmon cells.

5.2.8 Detection of PPARγ relocation

Cells were analyzed for relocation of PPARγ after treatment with rosiglitazone and applying immunofluorescence staining with anti-PPARγ antibody. No apparent relocation was observed in either sample. Green fluorescence signal was present throughout the cells, both in the nucleus and the cytosol. This was also the case for cells transfected with PPARγ plasmids, as discussed earlier. This could indicate either non-specific staining of primary antibody or

staining against endogenous PPAR. If the latter is the case, then there should not be any reason primary antibody do not stain transfected PPARγ, but as transfected cells are few, it is likely that these cells are hard to detect.

5.2.9 Protein detection

To be able to study transfected PPARγ on the protein level, we performed western blotting with an antiserum against the receptor. The antiserum did not seem to function in immunoblots against the cloned PPARγ construct, but against endogenous PPARγ or moreover non-specific. Proteins of approximately 50 and 60 kDa were identified (the molecular weight of PPARs corresponds to around 50 kDa). There was no increase in expression signal in transfected cells. All cell samples, including control and pmaxGFP^{TM}, expressed these signals. This strengthens the assumptions that the antiserum binds to endogenous PPARγ (correspond to band at 50 kDa) or that antiserum peptid was not PPARγ specific (band at 60 kDa).

CONCLUSION

- Our findings of the PPAR activity in Atlantic salmon head kidney cells agrees in many aspects with previous findings in mammalian cells.

- PPARs are expressed in higher levels in liver tissue than muscle and gills tisues and head kidney cell lines.

- Specific PPAR agonists could have a toxic effect on SHK-1 and ASK cells in the concentration 100μM.

- It was difficult to obtain high transfection efficiency in SHK-1 and ASK cells, it is necessary to optimalized the procedure and achieve higher tranfection efficiency to further study PPAR.

- SHK-1 and ASK cells are not suitable models for PPAR studies, because of the low expression levels and low transfection efficiency.

- Due to the low expression level and low transfection efficiency, it was not possible to determine the novel anti-PPARγ antibody level of specificity, with the head kidney salmon cells applied in this study.

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